

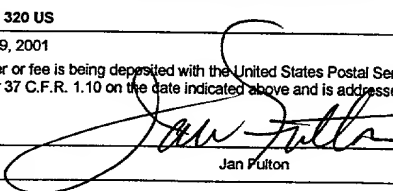
04-20-01

PCT

JC05 Rec'd PCT/PTO 1 9 APR 2001

1c846 U.S. PTO

FORM PTO-1390 DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (REV 1-98)		ATTORNEY'S DOCKET NO. 4050.000900
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/807877
		UNKNOWN
INTERNATIONAL APPLICATION NO. PCT/AU99/00968	INTERNATIONAL FILING DATE November 5, 1999	PRIORITY DATE CLAIMED November 6, 1998
TITLE OF INVENTION Regulation of Nitric Oxide Synthase Activity		
APPLICANT(S) FOR DO/EO/US David Ian Stapleton, Zhiping Chen, Belinda Joyce Michell, Bruce Ernest Kemp and Kenneth Ian Mitchelhill		
Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<ol style="list-style-type: none"> <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1). <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)). <ol style="list-style-type: none"> <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau); PCT cover page; 29 pages of text, Figure 1A-9 on 9 sheets, and 1 page of Search Report. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)). <ol style="list-style-type: none"> <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). <input type="checkbox"/> have been transmitted by the International Bureau. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. <input type="checkbox"/> have not been made and will not be made. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). 		
Items 11 to 16 below concern document(s) or information included: <ol style="list-style-type: none"> <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included and the fee is incorporated within the attached check. <input checked="" type="checkbox"/> A FIRST preliminary amendment (with Exhibits A, B and C); please calculate the filing fee based upon the claims in this amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. <input type="checkbox"/> A substitute specification. <input checked="" type="checkbox"/> Power of attorney and/or address letter. <input checked="" type="checkbox"/> Other items or information: 1. PCT Request; 2. International Search Report; 3. The references (3) cited in International Search Report; 4. PCT Demand; 5. International Preliminary Examination Report (IPER); 6. Notification Concerning Submission of Transmittal of Priority Document; and 7. Postcard 		

CERTIFICATE OF EXPRESS MAILING	
NUMBER	EL 522 496 320 US
DATE OF DEPOSIT:	April 19, 2001
I hereby certify that this paper or fee is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, DC 20231.	
 Jan Fulton	

U.S. APPLICATION NO. (if known) (37 CFR 1.5) UNKNOWN	INTERNATIONAL APPLICATION NO. PCT/AU/99/00968	ATTORNEY'S DOCKET NUMBER 4050.000900
---	--	---

09/807877

17. ☒ The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5)):
 Neither international preliminary examination fee (37 CFR 1.482)
 nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO
 and International Search Report not prepared by the EPO or JPO \$1,000.00

International preliminary examination fee (37 CFR 1.482) not paid to
 USPTO but International Search Report prepared by the EPO or JPO \$860.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO
 but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$760.00

international preliminary examination fee (37 CFR 1.482) paid to USPTO
 but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO
 and all claims satisfied provisions of PCT Article 33(1)-(4) \$ 100.00

ENTER APPROPRIATE BASIC FEE AMOUNT = \$ 1,000.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30
 months from the earliest claimed priority date (37 CFR 1.492(e)). \$ -0-

Claims	Number Filed	Number Extra	Rate	
Total Claims	24 - 20 =	4	x \$ 18.00	\$ 72.00
Independent Claims	4 - 3 =	1	x \$ 80.00	\$ 80.00
Multiple dependent claim(s) (if applicable)			+ \$260.00	\$ -0-
TOTAL OF ABOVE CALCULATIONS =				\$ 1,152.00
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity Statement no longer necessary				\$ 576.00
SUBTOTAL =				\$ 576.00
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$ -0-
TOTAL NATIONAL FEE =				\$ 576.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property)				\$ 40.00
TOTAL FEES ENCLOSED =				\$ 616.00
				Amount to be refunded: \$.00
				Charged \$.00

a. ☒ A check in the amount of \$616.00 to cover the above fees is enclosed.


b. ☐ Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0786/4050.000900. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Shelley P.M. Fussey
 WILLIAMS, MORGAN & AMERSON
 7676 Hillmont, Suite 250
 Houston, TX 77040
 (713) 934-7000
 Date: April 19, 2001



SIGNATURE

Shelley P.M. Fussey

NAME:

39,458

REGISTRATION NUMBER:

AMENDMENT

In the Specification:

Prior to page 1, where the text of the application begins, please delete the double-sided cover page from the PCT stage if necessary.

At page 1, after the title, in the current single-paragraph that constitutes the entire Section of the Application pertaining to the field of the invention and cross-reference to related applications, please delete the existing section and replace such deleted section with the following, two-paragraph replacement section:

The present application is a nationalization of International Patent Application PCT/AU99/00968, filed November 05, 1999, which claims priority to Australian Patent Application PP 6976, filed November 06, 1998.

This invention relates to the regulation of the activity of the enzyme nitric oxide synthase, and in particular to regulation of activity of endothelial and neuronal nitric oxide synthases. We have found that the phosphorylation of endothelial and neuronal nitric oxide synthases by several protein kinases, including protein kinase C and the AMP-activated protein kinase, regulates their activity.

After page 29, please start another page (30), and insert a section containing the following text of the Abstract, based upon the first cover page from the PCT application: - -

ABSTRACT

This invention relates to the regulation of the activity of the enzyme nitric oxide synthase, particularly the regulation of activity of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS and nNOS μ). The invention provides a method of identifying modulators of AMPK-mediated activation of eNOS, comprising testing putative modulators for

their ability to increase or decrease phosphorylation of eNOS depending on the calmodulin and calcium ion concentrations. The invention also provides a method of identifying modulators of AMPK-mediated inhibition of eNOS, comprising testing a putative modulator for its ability to decrease or increase AMPK-mediated phosphorylation of eNOS in the presence of limiting calcium ions. Preferably, specific phosphorylation of threonine 495 is assessed. The invention further provides a method of identifying modulators that either promote or inhibit phosphorylation of nNOS and nNOS_μ at Ser-1417. Compounds that activate the AMP-activated protein kinase are expected to be useful in the treatment of ischemic heart disease by promoting both glucose and fatty acid metabolism, as well as by increasing NOS activity to improve nutrient and oxygen supply to the myocytes and to reduce mechanical activity. These compounds also have utility in the treatment of pulmonary hypertension and in obstructive airways disease.

At the appropriate pages, prior to the text on each page, please delete the header that reads "WO 00/28076 PCT/AU99/00968" if necessary.

In the Claims:

After entry into the U.S. national stage, and assurance of a U.S. filing date, please revise the claims from the enclosed PCT application so that the rewritten claims read as follows:

5. (Amended) A method according to Claim 1, in which one or more of the following activities is additionally assessed:
 - (a) Effect on smooth muscle contraction;
 - (b) Effect on inotropic activity of the heart;
 - (b) Effect on chronotropic activity of the heart; or

(d) Effect on platelet function.

6. (Amended) A method according to Claim 1, in which the modulator is an activator, as herein defined.

8. (Amended) A method according to Claim 1, in which the modulator is an inhibitor, as herein defined.

9. (Amended) A method according to Claim 3, in which the modulator acts preferentially on non-neuronal cells.

10. (Amended) A method according to Claim 1, in which the modulator promotes the dephosphorylation of Ser-1177 and inhibits eNOS activity.

12. (Amended) A method according to Claim 1, in which the modulator promotes phosphorylation of nNOS or nNOS_μ at Ser-1417.

13. (Amended) A method according to Claim 1, in which the modulator promotes dephosphorylation of nNOS or nNOS_μ at Ser-1417.

Please add new claims 14-24, as follows:

14. (New) An antibody directed against eNOS, in which the eNOS is phosphorylated at Ser-1177 or at Thr-495.

15. (New) An antibody according to Claim 14, in which the eNOS is phosphorylated at Ser-1177.
16. (New) An antibody according to Claim 14, in which the eNOS is phosphorylated at Thr-495.
17. (New) An antibody according to Claim 14, in which the antibody is raised against a synthetic phosphopeptide comprising the sequence RIRTQSpFSLQER.
18. (New) An antibody according to Claim 14, in which the antibody is raised against a synthetic phosphopeptide comprising the sequence GITRKKTpFKEVANCV.
19. (New) An antibody according to Claim 14, which is a polyclonal antibody.
20. (New) An antibody according to Claim 14, which is a monoclonal antibody.
21. (New) An antibody according to Claim 14, labelled with a detectable marker.
22. (New) A method of detecting phosphorylation of eNOS, comprising the step of reacting a biological sample containing eNOS with an antibody according to claim 14.
23. (New) A method according to Claim 23, in which Ser-1177 is detected.

24. (New) A method according to Claim 23, in which phosphorylation at Thr-495 is detected.

REMARKS

I. Nationalization

This application represents the U.S. national stage of International Patent Application PCT/AU99/00968, filed November 05, 1999, which claims priority to Australian Patent Application PP 6976, filed November 06, 1998.

As the text of the International Application was transmitted by the International Bureau, an additional copy is not required to satisfy 35 U.S.C. § 371(c)(2). Nonetheless, for the Examiner's convenience, a copy of international application PCT/AU99/00968 is enclosed in the form of the published PCT Application WO 00/28076.

As there are no "substitute pages" within the written text, and after consultation with Australian counsel, Applicants' representative confirms that the text of the enclosed published PCT application corresponds to the text of the international application as filed. As the substitute pages of drawings were entered at the appropriate time during PCT examination, they will have been transmitted to the U.S. Office by the International Bureau.

Should formal amendments be necessary to conform to U.S. practice, Applicants seek to introduce such amendments into the present specification by, *e.g.*, deleting the PCT cover page, providing the Abstract as a separate page, and deleting the PCT header.

Priority is also properly claimed by an amendment at page 1.

II. National Stage Claims

After according a U.S. filing date, and before calculating the filing fee, entry of the foregoing claim amendments is respectfully requested. The changes to the pending claims are being made solely to conform to U.S. practice. New claims are being entered, beginning with claim 14. The new claims are fully supported by the PCT and priority application. None of the revised or new claims constitute new matter. The submission of revised claims does not represent abandonment of any of the subject matter of the claims in the international application.

III. Status of the Claims

At the conclusion of the PCT examination phase, claims 1-13 were pending (see IPER as well as PCT publication, both enclosed). The IPER finds each of claims 1-13 to have unity of invention, and is completely favorable regarding the novelty, inventive step and industrial applicability of all claims.

Presently, claims 5, 6, 8-10, 12 and 13 have been amended to render them singly dependent. No claims have been canceled. Claims 14-24 have been added, which are fully supported by the original specification.

Claims 1-24 are therefore in the case. For the convenience of the Examiner, a copy of the pending claims showing the revisions is included herewith as **Exhibit A**. A clean copy of the pending claims is included herewith as **Exhibit B**.

IV. Support for the Claims

Aside from removing the multiple dependencies in claims 5, 6, 8-10, 12 and 13, no changes to the pending claims have been introduced. Claims 1-13 thus represent those at the conclusion of PCT examination essentially in unamended form.

New claims 14-24 are supported throughout the specification. For example, exemplary support for claims 14-21 is particularly prominent at page 16, with exemplary support for claims 22-24 being prominent at page 17 and in the supporting figures. See also, page 4, lines 25-27, concerning the use of antibodies to phosphorylated at Ser-1177, confirming that this site is phosphorylated during ischemia.

Claims 17 and 18 are supported by the sequences in the original specification, although CRIRTQSpFSLQER reflects the addition of C at the N-terminus and GITRKKTpFKEVANC reflects deletion of V at the C-terminus. Those of ordinary skill in the art would understand the CRIRTQSpFSLQER sequence to properly reflect the addition of a cysteine at the N-terminus as cysteine is known in the art to be used for coupling peptides to keyhole limpet haemocyanin, described in the specification as being used in antibody generation. In terms of GITRKKTpFKEVANC, the deletion of valine from the C-terminus reflects the peptide actually used in immunization. Those of ordinary skill in the art would, in light of the present disclosure, understand the valine in question to be V504 of the primary eNOS sequence, and would therefore understand that the presence or absence of this valine would not be material to the generation of antibodies with specificity for the phosphorylated peptide as opposed to the dephosphorylated peptide. This is because the valine at position 504 is sufficiently removed from the threonine residue that becomes phosphorylated, *i.e.*, the threonine at position 495.

It will therefore be understood that no new matter is encompassed by any of the amended or newly presented claims.

V. Compliance with 37 C.F.R. § 1.121

Copies of the pending claims are attached hereto as **Exhibit A** and **Exhibit B**. In accordance with 37 C.F.R. § 1.121, the claims have been labeled as "(Amended)" or "(New)", where appropriate. **Exhibit A** provides a clean copy of the pending claims, whereas **Exhibit B** shows the changes with brackets and underlining.

The proper claim for priority has been timely introduced into the specification by amendment of the opening paragraph at page 1. A 199 word Abstract is also introduced into the specification by amendment as a separate page.

The amendments to the opening paragraph at page 1 of the specification and the abstract have been made as "Replacement Sections" in accordance with 37 C.F.R. §§ 1.121(b)(2), 1.77(b)(2) and 1.77(b)(10). This is proper under 37 C.F.R. §§ 1.121(b)(2)(i)(ii)(iii), as the specification contains section headings as provided in 37 C.F.R. § 1.77, and the amendments include the reference, replacement section in clean form and another version of the replacement section separate from the amendment marked up to show all changes (**Exhibit C**).

VI. Fees and Formalities

The national filing fee and claim fees are included herewith. The fees have been calculated after the present changes to remove the multiple dependencies in the claims. Any omitted fees should be deducted from Williams, Morgan & Amerson Deposit Account No. 50-0786/4050.000900.

Applicants are entitled to small entity status. An executed declaration to this effect is no longer required.

VII. Conclusion

The IPER issued for the international application finds all claims to have unity of invention. Applicants therefore urge that they define a unified invention for the purposes of examination in the U.S.

Importantly, the IPER also holds that all claims meet the requirements for industrial applicability, novelty and inventive step. This is compelling evidence that the present claims have utility and define a novel and non-obvious invention that should be progressed to allowance in the United States.

In light of the positive IPER, Applicants submit that the present case is in condition for allowance and such favorable action is respectfully requested. Should the Examiner have any questions or comments, a telephone call to the undersigned Applicants' representative is earnestly solicited.

Respectfully submitted,



Shelley P.M. Fussey
Reg. No. 39,458
Agent for Applicants

WILLIAMS, MORGAN & AMERSON, P.C.
7676 Hillmont, Suite 250
Houston, Texas, 77040
(713) 934-4079

Date: April 19, 2001

EXHIBIT A
PENDING CLAIMS

1. A method of identifying modulators of AMPK-mediated activation of a nitric oxide synthase enzyme selected from the group consisting of eNOS, nNOS and nNOS_μ, comprising the step of testing putative modulators for their ability to increase or decrease phosphorylation of the enzyme, said increase or decrease depending on the calmodulin and calcium ion concentrations.
2. A method according to claim 1, in which the specific phosphorylation of Ser-1177 is assessed in the presence of calcium and calmodulin.
3. A method of identifying modulators of AMPK-mediated inhibition of eNOS, comprising the step of testing a putative modulator for its ability to decrease or increase AMPK-mediated phosphorylation of eNOS in the presence of limiting calcium ions.
4. A method according to claim 3, in which the specific phosphorylation of Thr-495 is assessed.
5. (Amended) A method according to Claim 1, in which one or more of the following activities is additionally assessed:
 - (a) Effect on smooth muscle contraction;
 - (b) Effect on inotropic activity of the heart;
 - (b) Effect on chronotropic activity of the heart; or
 - (d) Effect on platelet function.
6. (Amended) A method according to Claim 1, in which the modulator is an activator, as herein defined.
7. A method according to Claim 6, in which the activator promotes both glucose metabolism and fatty acid metabolism.
8. (Amended) A method according to Claim 1, in which the modulator is an inhibitor, as herein defined.

9. (Amended) A method according to Claim 3, in which the modulator acts preferentially on non-neuronal cells.
10. (Amended) A method according to Claim 1, in which the modulator promotes the dephosphorylation of Ser-1177 and inhibits eNOS activity.
11. A method according to Claim 3, in which the modulator promotes the dephosphorylation of Thr-495 and stimulates eNOS activity.
12. (Amended) A method according to Claim 1, in which the modulator promotes phosphorylation of nNOS or nNOS μ at Ser-1417.
13. (Amended) A method according to Claim 1, in which the modulator promotes dephosphorylation of nNOS or nNOS μ at Ser-1417.
14. (New) An antibody directed against eNOS, in which the eNOS is phosphorylated at Ser-1177 or at Thr-495.
15. (New) An antibody according to Claim 14, in which the eNOS is phosphorylated at Ser-1177.
16. (New) An antibody according to Claim 14, in which the eNOS is phosphorylated at Thr-495.
17. (New) An antibody according to Claim 14, in which the antibody is raised against a synthetic phosphopeptide comprising the sequence RIRTQSpFSLQER.
18. (New) An antibody according to Claim 14, in which the antibody is raised against a synthetic phosphopeptide comprising the sequence GITRKKTpFKEVANCV.
19. (New) An antibody according to Claim 14, which is a polyclonal antibody.
20. (New) An antibody according to Claim 14, which is a monoclonal antibody.

21. (New) An antibody according to Claim 14, labelled with a detectable marker.
22. (New) A method of detecting phosphorylation of eNOS, comprising the step of reacting a biological sample containing eNOS with an antibody according to claim 14.
23. (New) A method according to Claim 23, in which Ser-1177 is detected.
24. (New) A method according to Claim 23, in which phosphorylation at Thr-495 is detected.

EXHIBIT B
PENDING CLAIMS

1. A method of identifying modulators of AMPK-mediated activation of a nitric oxide synthase enzyme selected from the group consisting of eNOS, nNOS and nNOS_μ, comprising the step of testing putative modulators for their ability to increase or decrease phosphorylation of the enzyme, said increase or decrease depending on the calmodulin and calcium ion concentrations.
2. A method according to claim 1, in which the specific phosphorylation of Ser-1177 is assessed in the presence of calcium and calmodulin.
3. A method of identifying modulators of AMPK-mediated inhibition of eNOS, comprising the step of testing a putative modulator for its ability to decrease or increase AMPK-mediated phosphorylation of eNOS in the presence of limiting calcium ions.
4. A method according to claim 3, in which the specific phosphorylation of Thr-495 is assessed.
5. (Amended) A method according to [any one of Claims 1 to 4] Claim 1, in which one or more of the following activities is additionally assessed:
 - (a) Effect on smooth muscle contraction;
 - (b) Effect on inotropic activity of the heart;
 - (b) Effect on chronotropic activity of the heart; or
 - (d) Effect on platelet function.
6. (Amended) A method according to [any one of Claims 1 to 5] Claim 1, in which the modulator is an activator, as herein defined.
7. A method according to Claim 6, in which the activator promotes both glucose metabolism and fatty acid metabolism.
8. (Amended) A method according to [any one of Claims 1 to 5] Claim 1, in which the modulator is an inhibitor, as herein defined.

9. (Amended) A method according to [any one of Claims 3 to 8] Claim 3, in which the modulator acts preferentially on non-neuronal cells.
10. (Amended) A method according to Claim 1 [or Claim 2], in which the modulator promotes the dephosphorylation of Ser-1177 and inhibits eNOS activity.
11. A method according to Claim 3, in which the modulator promotes the dephosphorylation of Thr-495 and stimulates eNOS activity.
12. (Amended) A method according to Claim 1 [or Claim 2], in which the modulator promotes phosphorylation of nNOS or nNOS μ at Ser-1417.
13. (Amended) A method according to Claim 1 [or Claim 2], in which the modulator promotes dephosphorylation of nNOS or nNOS μ at Ser-1417.
14. (New) An antibody directed against eNOS, in which the eNOS is phosphorylated at Ser-1177 or at Thr-495.
15. (New) An antibody according to Claim 14, in which the eNOS is phosphorylated at Ser-1177.
16. (New) An antibody according to Claim 14, in which the eNOS is phosphorylated at Thr-495.
17. (New) An antibody according to Claim 14, in which the antibody is raised against a synthetic phosphopeptide comprising the sequence RIRTQSpFSLQER.
18. (New) An antibody according to Claim 14, in which the antibody is raised against a synthetic phosphopeptide comprising the sequence GITRKKTpFKEVANCV.
19. (New) An antibody according to Claim 14, which is a polyclonal antibody.
20. (New) An antibody according to Claim 14, which is a monoclonal antibody.

21. (New) An antibody according to Claim 14, labelled with a detectable marker.
22. (New) A method of detecting phosphorylation of eNOS, comprising the step of reacting a biological sample containing eNOS with an antibody according to claim 14.
23. (New) A method according to Claim 23, in which Ser-1177 is detected.
24. (New) A method according to Claim 23, in which phosphorylation at Thr-495 is detected.

EXHIBIT C
REPLACEMENT SECTIONS

At page 1, after the title, in the current single-paragraph that constitutes the entire Section of the Application pertaining to the field of the invention and cross-reference to related applications, the additions are as shown:

The present application is a nationalization of International Patent Application PCT/AU99/00968, filed November 05, 1999, which claims priority to Australian Patent Application PP 6976, filed November 06, 1998.

This invention relates to the regulation of the activity of the enzyme nitric oxide synthase, and in particular to regulation of activity of endothelial and neuronal nitric oxide synthases. We have found that the phosphorylation of endothelial and neuronal nitric oxide synthases by several protein kinases, including protein kinase C and the AMP-activated protein kinase, regulates their activity.

At page 1, after the title, in the current single-paragraph that constitutes the entire Section of the Application pertaining to the field of the invention and cross-reference to related applications, the final text is as follows:

The present application is a nationalization of International Patent Application PCT/AU99/00968, filed November 05, 1999, which claims priority to Australian Patent Application PP 6976, filed November 06, 1998.

This invention relates to the regulation of the activity of the enzyme nitric oxide synthase, and in particular to regulation of activity of endothelial and neuronal nitric oxide synthases. We have found that the phosphorylation of endothelial and neuronal nitric oxide synthases by several protein kinases, including protein kinase C and the AMP-activated protein kinase, regulates their activity.

In the Section of the Application that forms the Abstract, the deletions and additions are as shown:

This invention relates to the regulation of the activity of the enzyme nitric oxide synthase, [and in particular to] particularly the regulation of activity of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS and nNOS μ). [According to a first aspect, the] The invention provides a method of identifying modulators of AMPK-mediated activation of eNOS, comprising [the step of] testing putative modulators for their ability to increase or decrease phosphorylation of eNOS depending on the calmodulin and calcium ion concentrations. [In an alternative aspect, the] The invention also provides a method of identifying modulators of AMPK-mediated inhibition of eNOS, comprising [the step of] testing a putative modulator for its ability to decrease or increase AMPK-mediated phosphorylation of eNOS in the presence of limiting calcium ions. Preferably, specific phosphorylation of threonine 495 is assessed. [According to a second aspect, the] The invention further provides a method of identifying modulators that either promote or inhibit phosphorylation of nNOS and nNOS μ at Ser-1417. Compounds that activate the AMP-activated protein kinase are expected to be useful in the treatment of ischemic heart disease by promoting both glucose and fatty acid metabolism, as well as by increasing NOS activity to improve nutrient and oxygen supply to the myocytes and to reduce mechanical activity. These compounds [would] also have utility in the treatment of pulmonary hypertension and in obstructive airways disease.

In the Section of the Application that forms the Abstract, the final text is as follows:

This invention relates to the regulation of the activity of the enzyme nitric oxide synthase, particularly the regulation of activity of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS and nNOS μ). The invention provides a method of identifying modulators of AMPK-mediated activation of eNOS, comprising testing putative modulators for their ability to increase or decrease phosphorylation of eNOS depending on the calmodulin and calcium ion concentrations. The invention also provides a method of identifying modulators of AMPK-mediated inhibition of eNOS, comprising testing a putative modulator for its ability to decrease or increase AMPK-mediated phosphorylation of eNOS in the presence of limiting calcium ions. Preferably, specific phosphorylation of threonine 495 is assessed. The invention further provides a method of identifying modulators that either promote or inhibit phosphorylation of nNOS and nNOS μ at Ser-1417. Compounds that activate the AMP-activated protein kinase are expected to be useful in the treatment of ischemic heart disease by promoting both glucose and fatty acid metabolism, as well as by increasing NOS activity to improve nutrient and oxygen supply to the myocytes and to reduce mechanical activity. These compounds also have utility in the treatment of pulmonary hypertension and in obstructive airways disease.

REGULATION OF NITRIC OXIDE SYNTHASE ACTIVITY

This invention relates to the regulation of the activity of the enzyme nitric oxide synthase, and in particular to regulation of activity of endothelial and neuronal nitric oxide synthases. We have found that the phosphorylation of endothelial and neuronal nitric oxide synthases by several protein kinases, including protein kinase C and the AMP-activated protein kinase, regulates their activity .

BACKGROUND OF THE INVENTION

Nitric oxide (NO) has recently been recognised as an important mediator of a very wide variety of cellular functions, and is present in most if not all mammalian cells (Moncada, S. and Higgs, A., 1993). It is implicated in a range of disorders, hypertension, hypocholesterolaemia, diabetes, heart failure, aging, inflammation, and the effects of cigarette smoking, and is especially important in vascular biology. It regulates systemic blood pressure as well as vascular remodelling (Rudic et al., 1998) and angiogenesis in response to tissue ischaemia (Murohara et al., 1998). NO is synthesised from the amino acid L-arginine by the enzyme nitric oxide synthase (NOS).

Three isoforms of NOS have been identified: neuronal NOS (nNOS), which is found in neuronal tissues and skeletal muscle (nNOS μ isoform); inducible NOS (iNOS), found in a very wide variety of mammalian tissues including activated macrophages, cardiac myocytes, glial cells and vascular smooth muscle cells; and endothelial NOS (eNOS), found in vascular endothelium, cardiac myocytes and blood platelets. Endothelial cells produce NO in response to

- 2 -

1 shear stress generated by the streaming of blood on the
2 endothelial layer.

3 The three isoforms of NO synthase have an amino
4 acid sequence identity of approximately 55%, with strong
5 sequence conservation in regions involved in catalysis.
6 For all three isoforms, the mechanism of NO synthesis
7 involves binding of the ubiquitous calcium regulatory
8 protein calmodulin (CaM) to the enzyme. However, the
9 conditions under which CaM is bound appear to be different
10 for iNOS, at least insofar as calcium concentration is
11 concerned. These three NOS enzymes have been intensively
12 studied, and the field has been recently reviewed; see for
13 example Michel and Feron (1997); Harrison (1997); and Mayer
14 and Hellens (1997). Although it was known from earlier
15 studies that eNOS could be multiply phosphorylated, the
16 mechanism of these phosphorylation events, including the
17 enzyme responsible for phosphorylation, and the role of
18 phosphorylation in modulation of eNOS function was not
19 known.

20 AMP-activated protein kinase (AMPK) is a
21 metabolic stress-sensing protein kinase which is known to
22 play an important role in the regulation of acetyl-CoA
23 carboxylase, leading to the acceleration of fatty acid
24 oxidation during vigorous exercise or ischaemia. AMPK is
25 well known as a regulator of lipid metabolism, and in
26 particular is known to have a role in cholesterol
27 synthesis, as reviewed in Hardie and Carling (1997). The
28 AMPK is also considered to play an important role in
29 exercise-enhanced glucose transport (Hayashi et al., (1998)
30 which is distinct from the insulin-mediated glucose uptake
31 mechanism. AMPK has mainly been studied in the liver,
32 heart and skeletal muscle. AMPK has been purified, and the
33 genes encoding the enzyme subunits were cloned (See
34 International Patent Applications

1 numbers PCT/GB94/01093 and PCT/US97/00270 and publication
2 WO97/25341).

3 The mammalian AMPK (Mitchelhill et al, 1994) is
4 related to the *Saccharomyces cerevisiae* SNF1 protein
5 kinase. It is required for the expression of glucose-
6 repressed genes in response to nutritional stress which
7 requires growth on alternative carbon sources (Celenza and
8 Carlson, 1986); both the mammalian and yeast kinases are
9 activated by upstream kinases (Hardie and Carling, 1997).
10 The AMPK is involved in metabolic stress responses through
11 phosphorylation at Ser-79 and concomitant inhibition of
12 acetyl-CoA carboxylase and HMG-CoA reductase (Hardie and
13 Carling, 1997). Multiple AMPK isoforms occur. They
14 comprise $\alpha\beta\gamma$ heterotrimers consisting of either $\alpha 1$ or $\alpha 2$
15 catalytic sub-units (Stapleton et al, 1996; Stapleton et
16 al, 1997a), together with the non-catalytic subunits β and
17 γ (Mitchelhill et al, 1994; Carling et al, 1994; Stapleton
18 et al, 1994), which are related to the yeast sip1p and
19 snf4p respectively.

20 The AMPK $\alpha 2$ sub-unit gene is on chromosome 1
21 (Beri et al., 1994), the $\alpha 1$ sub-unit gene is on
22 chromosome 5, the $\beta 1$ and $\gamma 1$ sub-unit genes are on
23 chromosome 12, the $\beta 2$ sub-unit gene is on chromosome 1, and
24 the $\gamma 2$ sub-unit gene is localised on chromosome 7
25 (Stapleton et al, 1997). A $\gamma 3$ gene has been detected using
26 an expressed sequence tag (EST) generated by genome
27 sequencing (Accession No AA178898).

28 One of the genes encoding eNOS is on
29 chromosome 7, close to the gene for the $\gamma 2$ sub-unit of
30 AMPK. Another gene encoding nNOS is found on
31 chromosome 12. (The human gene map; SEE
32 <http://www.ncbi.nlm.nih.gov/cgi->

1 bin/SCIENCE96/tsrch?QTEXT=nitric+oxide+synthase)

2 Recent work has shown that the AMPK in cardiac
3 and skeletal muscle is activated by vigorous exercise or by
4 ischaemic stress (Winder and Hardie, 1996; Vavvas *et al*,
5 1997; Kudo *et al*, 1995). This led us to investigate the
6 localization of the AMPK isoforms in these tissues. The
7 AMPK- α 2 isoform is present in capillary endothelial cells
8 in cardiac and skeletal muscle, and the AMPK- α 1 isoform
9 occurs in cardiac myocytes and vessels. The presence of
10 AMPK in endothelial cells led us to test bacterially-
11 expressed eNOS as a substrate, and we found that it is
12 readily phosphorylated by either AMPK- α 1 or AMPK- α 2.

13 We have now surprisingly found that the
14 AMP-activated protein kinase phosphorylates and regulates
15 endothelial NO synthase. We find that the AMPK
16 phosphorylates eNOS at two sites. In the presence of
17 calcium and calmodulin, Ser-1177 in the human sequence, and
18 Ser-1179 for the bovine sequence is phosphorylated in the
19 COOH-terminal tail of the enzyme, causing activation of
20 eNOS by shifting the calmodulin-dose dependence. In the
21 absence of added calcium and calmodulin, phosphorylation
22 also occurs at Thr-495 in the eNOS calmodulin-binding
23 sequence, and inhibits the enzyme. Ischaemia of the heart
24 causes activation of the AMPK and of eNOS, mimicking the
25 effects of phosphorylation at Ser-1177. Phosphopeptide-
26 specific antibodies to phosphorylated Ser-1177 were used to
27 confirm that this site was phosphorylated during ischaemia.
28 Our results are of special interest because they identify a
29 link between metabolic stress, which reduces ATP and
30 increases AMP, and signalling through eNOS to control
31 nutrient availability (via arterial vasodilation) as well
32 as suppressing myocardial contraction. This couples the
33 metabolic status of endothelial cells and myocytes with the

- 5 -

1 vascular supply and mechanical demands. Our results
2 provide a new insight into the post-translational
3 regulation of eNOS which is of particular significance for
4 the cardiovascular and skeletal muscle field. In addition,
5 similarities in structure and behaviour between eNOS and
6 nNOS have been identified, enabling us to identify
7 modulators of the activity of both these enzymes.

8

9 SUMMARY OF THE INVENTION

10 According to a first aspect, the invention
11 provides a method of identifying modulators of AMPK-
12 mediated activation of a nitric oxide synthase enzyme
13 selected from the group consisting of eNOS, nNOS and nNOS μ ,
14 comprising the step of testing the ability of putative
15 modulators to increase or decrease phosphorylation of the
16 enzyme; said increase or decrease depending on the
17 calmodulin and calcium ion concentrations.

18 Preferably the specific phosphorylation of
19 Ser-1177 is assessed in the presence of calcium and
20 calmodulin.

21 In an alternative aspect, the invention provides
22 a method of identifying modulators of AMPK-mediated
23 inhibition of eNOS, comprising the step of testing a
24 putative modulator for its ability to decrease or increase
25 AMPK-mediated phosphorylation of eNOS in the presence of
26 limiting calcium ions. Preferably specific phosphorylation
27 of Thr-495 is assessed.

28 Compounds able to increase phosphorylation of
29 Ser-1177 or decrease phosphorylation of Thr-495 are
30 referred to herein as activators, and compounds able to
31 decrease phosphorylation of Ser-1177 or increase
32 phosphorylation of Thr-495 are referred to as inhibitors.

- 6 -

1 In both aspects of the invention, one or more of
2 the following activities may optionally be additionally
3 assessed for each putative activator or inhibitor
4 identified by the method of the invention:

5 (a) Effect on smooth muscle contraction;

6 (b) Effect on inotropic activity of the
7 heart;

8 (c) Effect on chronotropic activity of the
9 heart; and

10 (d) Effect on platelet function.

11 It is expected that because the phosphorylation
12 site equivalent to Thr-495 in the eNOS calmodulin-binding
13 site is absent from the neuronal form of NOS, inhibitors
14 and activators identified by the method of the invention
15 will have at least some degree of tissue specificity.

16 Compounds that activate the AMP-activated protein
17 kinase are expected to be useful in ischaemic heart disease
18 by promoting both glucose and fatty acid metabolism, as
19 well as by increasing NOS activity to improve nutrient and
20 oxygen supply to the myocytes and to reduce mechanical
21 activity. These compounds would also have utility in
22 pulmonary hypertension and in obstructive airways disease.

23 For the purposes of this specification it will be
24 clearly understood that the word "comprising" means
25 "including but not limited to", and that the word
26 "comprises" has a corresponding meaning.

27
28 BRIEF DESCRIPTION OF THE FIGURES

29 Figure 1 shows immunofluorescence localization of
30 AMPK- α 2 in the heart and in the tibialis anterior muscle.

- 7 -

1 Panel A shows a negative control section of rat
2 heart stained with control rabbit IgG and control mouse
3 IgG, together with anti-rabbit-FITC and anti-mouse-Texas
4 Red.

5 Panel B shows a section of rat heart stained with
6 affinity-purified rabbit polyclonal antibody against
7 AMPK- α 2 (491-514) and anti-rabbit-FITC.

8 Panel C shows the same section as Panel B,
9 stained with a monoclonal antibody against rat endothelium
10 recA-1 and anti-mouse-Texas Red.

11 Panel D shows the overlay of Panels B and C.
12 Colocalization can be seen by the coincidence of staining.
13 The arrows highlight specific endothelial cells that are
14 stained by both antibodies.

15 Panel E shows a negative control section of rat
16 tibialis anterior muscle stained with control rabbit IgG
17 and control mouse-IgG, together with anti-rabbit-FITC and
18 anti-mouse-Texas Red.

19 Panel F shows a section stained with affinity-
20 purified rabbit polyclonal antibody against AMPK- α 2
21 (491-514) and anti-rabbit-FITC.

22 Panel G shows the same section as in Panel B,
23 stained with a monoclonal antibody against rat endothelium
24 recA-1 and anti-mouse-Texas Red.

25 Panel H shows the overlay of Panels E and F.
26 Colocalization can be seen by the coincidence of staining.

27 Figure 2 illustrates phosphorylation of
28 recombinant eNOS by AMPK.

29 Top panel: eNOS was incubated with rat liver
30 AMPK- α 1 and [γ - 32 P] ATP.

31 Lane 1: Coomassie-stained SDS-PAGE;

- 8 -

Lane 2: Autoradiograph.

Lower panel: ^{32}P -tryptic phosphopeptide map of eNOS.

Figure 3 shows the effect of phosphorylation of eNOS by the AMPK with or without added Ca^{2+} -CaM. Rat heart eNOS purified by 2',5'-ADP-Sepharose affinity chromatography was phosphorylated by AMPK in the presence of $0.8 \mu\text{M}$ CaM/ $3.2 \mu\text{M}$ Ca^{2+} (closed circles), in the absence of Ca^{2+} -CaM (closed triangles) and without AMPK (open squares). After phosphorylation, samples were diluted and eNOS activity was measured. The lower panels show phosphopeptide maps for rat heart eNOS phosphorylated in the presence and absence of added Ca^{2+} -CaM.

Figure 4 shows the effect of ischaemia on the activities of AMPK- $\alpha 1$, AMPK- $\alpha 2$ and eNOS.

Panel A shows the results of immunoprecipitation using antibody specific for AMPK- $\alpha 1$ and AMPK- $\alpha 2$, assayed using the SAMS peptide substrate. Results shown are mean \pm SEM for $n=5$.

Panel B shows eNOS activity measured at 500 nM CaM.

Panel C shows eNOS activities with full CaM-dose responses for a representative experiment. Ischaemia time points: 0 min (open squares), 1 min (closed diamonds), 10 min (closed circles) and 20 min (open triangles). The results of 4 replicates were the same, except that in one case the 20 min ischaemia eNOS CaM-dependence remained the same as for 10 min.

Figure 5 shows a comparison of NOS sequences. Phosphorylation site sequences for eNOS and nNOS are indicated in a schematic model of NOS. Sequences from the CaM-binding region (around the Thr-495 phosphorylation site

1 in eNOS) and for the COOH-terminal tail (around the
2 Ser-1177 phosphorylation site in eNOS) are shown.

3 Figure 6 shows the effect of treatment of bovine
4 aortic endothelial cells with phorbol ester (PMA) and
5 okadaic acid on eNOS activity (upper pane) and the
6 phosphorylation at Ser-1177 and Thr-495 (lower panel).

7 Figure 7 shows the effect of treatment of bovine
8 aortic endothelial cells with 3-isobutyl-1-methylxanthine
9 (IBMX) and calyculin A on the phosphorylation at Ser-1177
10 and Thr-495.

11 Figure 8 shows a summary illustration of the
12 regulation of eNOS by phosphorylation at Thr-495 and Ser-
13 1177, mediated by protein kinases PKC, AMPK and Akt.
14 Reversal of the phosphorylation at these sites is mediated
15 by protein phosphatases PP1 and PP2A in response to
16 treating the cells with IBMX and PMA respectively.

17 Figure 9 shows the effect of a 30 second bicycle
18 sprint exercise on nNOS phosphorylation in human muscle.
19 The nNOS was extracted from biopsy material and probed for
20 phosphorylation at Ser-1417 using an anti-phosphopeptide
21 antibody. The left panel shows an immunoblot, and the
22 right panel shows quantitative analysis of 5 individuals.

23

24 DETAILED DESCRIPTION OF THE INVENTION

25 The invention will now be described in detail by
26 way of reference only to the following non-limiting
27 examples and to the figures.

28 We have surprisingly found that in the presence
29 of Ca^{2+} -calmodulin (CaM) eNOS is phosphorylated by AMPK at
30 Ser-1177, resulting in activation, whereas phosphorylation
31 of eNOS in the absence of Ca^{2+} occurs predominantly at
32 Thr-495, a site in the CaM-binding sequence, resulting in

- 10 -

1 inhibition. It had previously been considered that
2 phosphorylation was solely inhibitory. We have also found
3 that ischaemia of the heart leads to rapid activation of
4 both isoforms of the metabolic stress-sensing enzyme AMPK
5 and eNOS. These data suggest that the AMPK may operate an
6 "inside-out" signalling pathway that leads to arterial
7 vasodilation and reduced myocardial contraction, so
8 coupling the metabolic status of endothelial cells and
9 myocytes with the vascular supply and mechanical activity.

10
11 Example 1 Immunofluorescence Localisation of AMPK- α 2
12 in Heart and Skeletal Muscle

13 Confocal immunofluorescence microscopy using
14 affinity-purified rabbit polyclonal antibody directed
15 against AMPK- α 2 (antibody 491-414. Staining with
16 fluorescence-labelled anti-rabbit antibody showed that the
17 α 2 isoform is found predominantly in capillary endothelial
18 cells in both cardiac muscle and skeletal muscle, while
19 cardiac myocytes and blood vessels showed intense but
20 diffuse staining for the α 1 AMPK isoform. In skeletal
21 muscle, the α 2 isoform was found in endothelial cells of
22 capillaries, and in fast-twitch muscle fibres, whereas the
23 α 1 isoform was found in Type I aerobic fibres.

24 Localisation of AMPK- α 2 in capillary endothelial cells in
25 both cardiac and skeletal muscle is illustrated in
26 Figure 1.

27
28 Example 2 AMPK Phosphorylates Recombinant eNOS

29 Bacterially expressed eNOS, coexpressed with CaM
30 by the method of Rodriguez-Crespo et al (1996), was
31 phosphorylated by either AMPK- α 1, as shown in Figure 2 top

- 11 -

1 panel, or AMPK- α 2. Recombinant eNOS phosphorylation by
2 immunoprecipitated AMPK- α 2 was detected. Since we have
3 been unable to purify high specific activity AMPK- α 2, no
4 further characterisation of eNOS regulation or the sites of
5 phosphorylation by the α 2 isoform was undertaken. Analysis
6 of the phosphorylation sites in eNOS following tryptic
7 digestion revealed four phosphopeptides generated from
8 three separate sites (Figure 2 bottom panel, A, A', B, C).
9 Identification of phosphorylation sites by mass
10 spectrometry and Edman sequencing, using the modified
11 method described by (Mitchelhill and Kemp, 1999), revealed
12 that Ser-1177 was the most prominent phosphorylation site,
13 as shown in Figure 2 bottom panel, A, A', and that its
14 phosphorylation was dependent on the presence of Ca^{2+} -CaM.

15 Phosphopeptide isolation from in-gel tryptic
16 digests was carried out as described by Mitchelhill et al
17 (1997a). Greater than 98% of the radioactivity was
18 recovered from the gel. Peptides isolated and characterized
19 by mass spectrometry and Edman sequencing are set out in
20 Table 1.

Table 1
Phosphopeptides Isolated from In-Gel tryptic Digests

Observed Mass	Phosphopeptide	Sequence	Calculated Mass
1440.0	B	KKTFKEVANAVK	1361.1 (*1441.7)
1174.1	A	TQXFSLQER	1094.5 (*1174.5)
1445.6	A'	IRTQXFSLQER	1363.7 (*1443.7)
1176.7	C	pCLGSLVFPR	1095.6 (*1175.6)

where: "pc" denotes pyridylethyl cysteine.

• denotes calculated mass of mono-phosphorylated peptide.

1 The location of the phosphorylation site in
2 peptide A, TQXFSLQER, was identified by ³²P-phosphate
3 release sequencing (Mitchelhill *et al*, 1997a). eNOS
4 phosphorylated by the AMPK- α 1 was no longer recognized by
5 the antibody to the eNOS COOH-terminal tail; nor was it
6 eluted from the ADP-Sepharose affinity column by
7 100 mM NADPH. These properties prevented the direct
8 confirmation of Ser-1177 phosphorylation *in situ*. This is
9 illustrated in Venema *et al*, 1996.

10 A second site, Thr-495, was phosphorylated in the
11 absence of Ca²⁺-CaM or when EGTA was present. This is
12 illustrated in Figure 2 bottom panel, B. This residue is
13 located in the CaM-binding sequence,

14 TRKKT⁴⁹⁵FKEVANAVKISASLM,

15 between the oxidase and reductase domains of eNOS (Venema
16 *et al*, 1996). Ser-101 in the N-terminal region of eNOS was
17 identified as a minor site of phosphorylation (Figure 2
18 bottom panel, C).

19 Synthetic peptides containing Thr-495 or Ser-1177
20 were readily phosphorylated by AMPK, with similar kinetic
21 values to the SAMS peptide substrate. The peptide
22 containing Thr-495, GTGITRKKTFKEVANAVK, was phosphorylated
23 with a Km of 39 \pm 10 μ M and a Vmax of
24 6.7 \pm 0.6 μ mol/min/mg, whereas the peptide containing
25 Ser-1177, RIRTQSFSLQERQLRG was phosphorylated with a Km of
26 54 \pm 6 μ M and a Vmax of 5.8 \pm 0.3 μ mol/min/mg. These are
27 comparable to results obtained using the well-characterized
28 SAMS peptide substrate, which has a Km 33 \pm 3 μ M and a Vmax
29 of 8.1 \pm 1.5 μ mol/min/mg (Michell *et al*, 1996). The *in*
30 *vitro* phosphorylation of the peptides confirms the
31 identification sites of phosphorylation.

- 14 -

1 Example 3 Effect of Ca^{2+} -CaM on Phosphorylation of
2 eNOS by AMPK

3 The eNOS activity was determined by measuring
4 L-[^3H]-citrulline production, using the method of Balligand
5 et al, 1995. The recombinant eNOS was coexpressed with
6 CaM, as described by Rodriguez-Crespo and Ortiz de
7 Montellano, 1996. Partially purified rat heart eNOS
8 contained some Ca^{2+} -CaM. In the absence of added EGTA, CaM
9 dependence was observed at 0-100 nM added CaM. In order to
10 investigate the changes in NOS activity with
11 phosphorylation in the absence and presence of Ca^{2+} -CaM,
12 EGTA buffering was used to achieve CaM dose response curves
13 in the range 0-1 μM . Routinely, 7-15 μM EGTA was added to
14 make eNOS activity dependent upon added CaM. Where
15 Ca^{2+} -CaM was used in the phosphorylation reaction prior to
16 eNOS assay, the samples were either diluted so that the
17 extra Ca^{2+} -CaM was negligible, or the indicated
18 concentrations represent total final concentrations of
19 added Ca^{2+} -CaM.

20 Cardiac eNOS was partially purified as follows.
21 Twenty rat hearts were homogenised in 80 ml of ice-cold
22 buffer A [50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA,
23 1 mM DTT, 50 mM NaF, 5 mM Na Pyrophosphate,
24 10 $\mu\text{g/ml}$ Trypsin inhibitor, 2 $\mu\text{g/ml}$ Aprotinin,
25 1 mM Benzamidine, 1 mM PMSF, 10% Glycerol, 1% Triton-X-
26 100]. The homogenate was put on ice for 30 min and
27 centrifuged at 16,000 x g for 30 min. The supernatant was
28 incubated with 2 ml of 2',5'-ADP-Sepharose (Bredt and
29 Snyder, 1990). The suspension was incubated for one hour
30 before washing in a fritted column, with 20 ml of buffer A
31 and 20 ml of buffer A containing 0.5 M NaCl, and then with
32 20 ml of buffer B [50 mM Tris-HCl, pH 7.5, 1 mM DTT,
33 10% Glycerol, 0.1% Triton-X-100]. eNOS was eluted with
34 buffer B containing 2 mM NADPH, then subjected to

- 15 -

centrifugal filtration (ULTRAFREE-MC MILLIPORE) to remove NADPH. Immunoblotting was used for selective detection of eNOS rather than nNOS.

Phosphorylation of eNOS by AMPK in the presence of Ca^{2+} -CaM resulted in activation, but CaM-dependence was retained, as shown in Figure 3 top panel. Activation shifted the dose response curve for CaM to the left. Phosphopeptide mapping revealed that activation of eNOS was correlated with phosphorylation of Ser-1177 but not of Thr-495, as shown in Figure 3 lower panel. Phosphorylation without added Ca^{2+} -CaM enhanced Thr-495 phosphorylation, suppressed Ser-1177 phosphorylation, and inhibited eNOS activity (Figure 3 top panel). The inhibition of eNOS activity by Thr-495 phosphorylation is consistent with earlier reports that phosphorylation of synthetic peptides corresponding to this region by protein kinase C inhibits CaM-binding (Matsubara *et al*, 1996). Similar results have been reported for nNOS (Loche *et al*, 1997).

Example 4 Effect of Ischaemia on Activities of
AMPK- α 1, AMPK- α 2 and eNOS

Langendorf preparations of isolated perfused rat heart were subjected to ischaemia according to the method of Kudo *et al* (1995). AMPK- α 1 and AMPK- α 2 isoforms were immunoprecipitated using α 2 (490-516) or α 1 (231-251) antibodies, and assayed using the SAMS peptide substrate (Michell *et al*, 1996; Hardie and Carling, 1997). eNOS activity was measured as described in Example 3. The results are shown in Figure 4. Both α 1 and α 2 isoforms are activated, as shown in Figure 4A, indicating that AMPK is activated in both capillary endothelial cells, which have predominantly the α 2 isoform, and in cardiac myocytes,

- 16 -

1 which have predominantly the $\alpha 1$ isoform. AMPK activation
2 during ischaemia is also accompanied by eNOS activation and
3 changes in the CaM dependence, as shown in Figures 4B and
4 4C, mimicking the effect of eNOS phosphorylation by AMPK *in*
5 *vitro*, as shown in Figure 3.

6 Polyclonal antibodies were raised against
7 synthetic phosphopeptides based on the eNOS sequence:
8 RIRTQSpFSLQER and GITRKKTpFKEVANCV. Rabbits were immunized
9 with phosphopeptides coupled to keyhole limpet haemocyanin
10 and then emulsified in Freund's complete adjuvant, using
11 conventional methods. The antibodies were purified using
12 the corresponding phosphopeptide affinity columns after
13 thorough preclearing with dephosphopeptide affinity
14 columns. The specificity of the purified antibodies was
15 confirmed using both EIA and immunoblotting, confirming
16 that they did not recognize recombinant dephospho-eNOS.

17 Using the anti-phosphopeptide antibodies to Ser-
18 1177 and Thr-495 phosphorylation sites we observed that
19 phosphorylation of Ser-1177 was increased approximately 3-
20 fold by ischaemia, but that there was no detectable change
21 in the Thr-495 phosphorylation under these conditions.
22 Heart muscle contains eNOS in both capillary endothelial
23 cells and cardiac myocytes (Balligand et al, 1995), with
24 low levels of the nNOS μ isoform (Silvagno et al, 1996).

25 The sequences of the three types of NOS are
26 compared in Figure 5, which shows the CaM-binding region
27 and the C-terminal tail. In nNOS Ser-1417 corresponds to
28 eNOS Ser-1177, whereas iNOS is truncated, and has a Glu in
29 this region. Both iNOS and nNOS lack a phosphorylatable
30 residue equivalent to Thr-495 in the CaM-binding region.

1

2

3 Example 5 Effect of Stimulation of Protein Kinase C on
4 eNOS Phosphorylation

5 Bovine aortic endothelial cells cultured in 0.1%
6 foetal calf serum for 20 hours (serum starved) were
7 subjected to treatment with the protein kinase C activator
8 0.1 μ M phorbol-12-myristate-13-acetate (PMA) for 5 min.
9 PMA treatment increased the phosphorylation of eNOS at Thr-
10 495 and decreased the phosphorylation at Ser-1177, as
11 measured using anti-phosphopeptide specific antibodies.
12 The antibodies used were the same as those described in
13 Example 4. The results are shown in Figure 6. In cells
14 cultured in medium without calcium we observed a 4-fold
15 decrease in Ser-1177 phosphorylation. Furthermore, when
16 cells were incubated in standard medium containing calcium
17 addition of the calcium ionophore A23187 (10 μ M for 90
18 seconds) increased Ser-1177 phosphorylation by a further 7-
19 fold. Preincubation of the cells with 0.5 μ M okadaic acid
20 prevented the dephosphorylation of Ser-1177 by PMA
21 treatment, and greatly augmented the phosphorylation of
22 Thr-495 (Results mean \pm SEM, n =6). Since okadaic acid
23 inhibits protein phosphatase PP2A, the results indicate
24 that PP2A is responsible for dephosphorylation of Ser-1177.
25 The changes observed in Thr-495 and Ser-1177
26 phosphorylation in response to treatment with PMA and
27 okadaic acid were reflected in the activity of eNOS.
28 Increased phosphorylation of Thr-495 with PMA or PMA plus
29 okadaic acid was associated with reduced eNOS activity.
30 Okadaic acid alone increased Ser-1177 phosphorylation
31 without altering Thr-495 phosphorylation, and was
32 associated with increased eNOS activity (Figure 6 upper
33 panel).

Example 6 Effect of Inhibition of Phosphodiesterase
and Phosphatase on the Phosphorylation of eNOS

The experimental details were similar to those for Example 5. Bovine aortic endothelial cells were preincubated with or without 10 nM of the phosphatase inhibitor calyculin A for 10 min, and then incubated with or without 0.5 mM of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) for 5 min. As shown in Figure 7, IBMX treatment caused enhanced phosphorylation of Ser-1177 and dephosphorylation of Thr-495. Preincubation with calyculin A prevented the dephosphorylation of Thr-495. (Results mean \pm SEM, n =6). Since calyculin A inhibits protein phosphatase PP1, the results indicate that PP1 is responsible for dephosphorylation of Thr-495.

DISCUSSION

Since the identification of the Ser-1177 phosphorylation site by the present inventors, it has been recognized that other protein kinases phosphorylate at this site. In particular, the protein kinase Akt (also named PKB) phosphorylates Ser-1177 in response to stimulation of endothelial cells by vascular endothelial growth factor (VEGF) (Fulton et al. 1999; Michell et al., 1999) or to fluid shear stress (Dimmeler et al., 1999; Gallis et al., 1999). In the study by Gallis et al. (1999) it was reported that fluid shear stress stimulated the phosphorylation of Ser-116 in the sequence KLQTRPSPGPPPA. Neither the kinase responsible nor the functional effects of phosphorylation of this site on eNOS has yet been identified. This phosphorylation site is present in the oxidase domain.

We have found that phosphorylation of eNOS at Thr-495 by protein kinase C occurs in endothelial cells that have been serum starved and incubated in calcium-free medium in the presence of the phorbol ester PMA. There is a reciprocal relationship between phosphorylation at Ser-1177 and Thr-495 in endothelial cells. Protein kinase C phosphorylates both sites *in vitro*, but stimulation of protein kinase C in endothelial cells with phorbol ester causes enhanced Thr-495 phosphorylation but marked phosphorylation of Ser-1177. The dephosphorylation of Ser-1177 is prevented by okadaic acid but not by calyculin A, indicating that phosphatase PP2A is responsible. Okadaic acid also greatly enhances the phosphorylation of Thr-495 in response to phorbol ester. Thrombin, which also acts via protein kinase C, stimulates phosphorylation of Thr-495 and dephosphorylation of Ser-1177.

In contrast, treatment of endothelial cells with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) causes a pronounced dephosphorylation of eNOS at Thr-495 and enhanced Ser-1177 phosphorylation. Dephosphorylation of Thr-495 in response to IBMX is blocked by treatment with calyculin A, suggesting that phosphatase PP1 is responsible for Thr-495 dephosphorylation.

These relationships are summarised in Figure 8. We find that exercise of skeletal muscle results in the phosphorylation of nNOS μ at Ser-1417, the site corresponding to Ser-1177 in eNOS (see Figure 5). Electrical stimulation of rat extensor digitorum longus (EDL) muscle was found to activate the AMPK, to phosphorylate acetyl CoA carboxylase at Ser-79 (the inhibitory site), and to phosphorylate nNOS μ at Ser 1417. Similarly, in biopsies of human skeletal muscle following vigorous exercise, such as a 30-second bicycle sprint, there is a 10-fold increase in phosphorylation on Ser-79 in

- 20 -

1 acetyl CoA carboxylase and a 7.5-fold increase in nNOS μ
2 phosphorylation at Ser-1417 (see Figure 9).

3 Endothelially-derived NO has a critical role in
4 preventing premature platelet adhesion and aggregation that
5 leads to thrombus formation (Radomski and Moncada, 1993).
6 There is evidence that the protective effects of elevated
7 high-density lipoprotein (HDL) on the cardiovascular system
8 may be mediated via increased platelet NO production.
9 Apolipoprotein E, a component of HDL, acts on a receptor
10 (apoER2) present in platelets to stimulate the NO signal
11 transduction pathway (Riddell et al., 1997; Riddell and
12 Owen, 1999).

13 Activation of eNOS by phosphorylation of its
14 COOH-terminal tail gives new insight into eNOS
15 autoinhibition. The increased activity and shift in the
16 CaM-dose dependence with phosphorylation at Ser-1177
17 suggest that in eNOS, and perhaps nNOS, the COOH-terminal
18 tails act as partial autoregulatory sequences analogous to
19 those in the CaM-dependent protein kinases (Kemp and
20 Pearson, 1991; Kobe et al, 1996).

21 The COOH-terminal tail of eNOS is only fully
22 accessible to the AMPK when Ca²⁺-CaM is bound, consistent
23 with this region being buried in the absence of CaM. As
24 can be seen from Figure 5, there is a high level of
25 similarity between eNOS and nNOS in their COOH-terminal
26 tails, whereas iNOS is distinct. It is known that the iNOS
27 CaM-binding, which is characterised by a low
28 Ca²⁺-dependence, requires both the canonical CaM-binding
29 sequence and distal residues in the COOH-terminus that
30 cannot be satisfied by nNOS chimeras (Ruan et al, 1996).
31 Without wishing to be bound by any proposed mechanism, we
32 believe that eNOS and nNOS are autoinhibited by their
33 COOH-terminal tails, requiring a two-stage activation

- 21 -

1 process for full activity with both CaM-binding and
2 phosphorylation in the tail, whereas iNOS requires only CaM
3 binding. Recently Salerno et al (1997) proposed that an
4 insert sequence in the FMN-binding domain may also be
5 important in autoregulation.

6 Previous studies have shown that eNOS may be
7 phosphorylated both *in vitro* and *in vivo*, but the precise
8 sites of phosphorylation and the function of the
9 phosphorylation events have not hitherto been fully
10 characterized (reviewed in Michel and Feron, 1997). eNOS
11 is the first example of an enzyme activated by AMPK to be
12 identified, and is also unusual because phosphorylation can
13 lead to either activation or inhibition, depending on the
14 availability of Ca²⁺-CaM. Other enzymes, notably the
15 cyclin-dependent protein kinases, are activated or
16 inhibited by phosphorylation, but this is catalysed by
17 different protein kinases. Protein kinase C phosphorylates
18 Thr-495 in eNOS, demonstrating intersecting regulatory
19 pathways acting on eNOS by phosphorylation of Thr-495 or
20 Ser-1177. It is also possible that persistent activation
21 of protein kinase C, for example in response to
22 hyperglycaemia induced by diabetes, could chronically
23 suppress phosphorylation of eNOS at Ser-1177, and thereby
24 reduce its activity.

25 The regulation of eNOS by AMPK extends the
26 conceptual relationship between the yeast snflp kinase and
27 the AMPK. Snflp kinase modulates the supply of glucose from
28 the environment by secreting invertase whereas the
29 mammalian AMPK integrates metabolic stress signalling with
30 the control of the circulatory system. Thus intracellular
31 metabolic stress signals within endothelial cells and
32 myocytes can elicit improved nutrient supply and suppress
33 mechanical activity of the muscle.

- 22 -

1 It will be apparent to the person skilled in the
2 art that while the invention has been described in some
3 detail for the purposes of clarity and understanding,
4 various modifications and alterations to the embodiments
5 and methods described herein may be made without departing
6 from the scope of the inventive concept disclosed in this
7 specification.

8 References cited herein are listed on the
9 following pages, and are incorporated herein by this
10 reference.

11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000
1001
1002
1003
1004
1005
1006
1007
1008
1009
1010
1011
1012
1013
1014
1015
1016
1017
1018
1019
1020
1021
1022
1023
1024
1025
1026
1027
1028
1029
1030
1031
1032
1033
1034
1035
1036
1037
1038
1039
1040
1041
1042
1043
1044
1045
1046
1047
1048
1049
1050
1051
1052
1053
1054
1055
1056
1057
1058
1059
1060
1061
1062
1063
1064
1065
1066
1067
1068
1069
1070
1071
1072
1073
1074
1075
1076
1077
1078
1079
1080
1081
1082
1083
1084
1085
1086
1087
1088
1089
1090
1091
1092
1093
1094
1095
1096
1097
1098
1099
1100
1101
1102
1103
1104
1105
1106
1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122
1123
1124
1125
1126
1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1145
1146
1147
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
1161
1162
1163
1164
1165
1166
1167
1168
1169
1170
1171
1172
1173
1174
1175
1176
1177
1178
1179
1180
1181
1182
1183
1184
1185
1186
1187
1188
1189
1190
1191
1192
1193
1194
1195
1196
1197
1198
1199
1200
1201
1202
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
1213
1214
1215
1216
1217
1218
1219
1220
1221
1222
1223
1224
1225
1226
1227
1228
1229
1230
1231
1232
1233
1234
1235
1236
1237
1238
1239
1240
1241
1242
1243
1244
1245
1246
1247
1248
1249
1250
1251
1252
1253
1254
1255
1256
1257
1258
1259
1260
1261
1262
1263
1264
1265
1266
1267
1268
1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281
1282
1283
1284
1285
1286
1287
1288
1289
1290
1291
1292
1293
1294
1295
1296
1297
1298
1299
1300
1301
1302
1303
1304
1305
1306
1307
1308
1309
1310
1311
1312
1313
1314
1315
1316
1317
1318
1319
1320
1321
1322
1323
1324
1325
1326
1327
1328
1329
1330
1331
1332
1333
1334
1335
1336
1337
1338
1339
1340
1341
1342
1343
1344
1345
1346
1347
1348
1349
1350
1351
1352
1353
1354
1355
1356
1357
1358
1359
1360
1361
1362
1363
1364
1365
1366
1367
1368
1369
1370
1371
1372
1373
1374
1375
1376
1377
1378
1379
1380
1381
1382
1383
1384
1385
1386
1387
1388
1389
1390
1391
1392
1393
1394
1395
1396
1397
1398
1399
1400
1401
1402
1403
1404
1405
1406
1407
1408
1409
1410
1411
1412
1413
1414
1415
1416
1417
1418
1419
1420
1421
1422
1423
1424
1425
1426
1427
1428
1429
1430
1431
1432
1433
1434
1435
1436
1437
1438
1439
1440
1441
1442
1443
1444
1445
1446
1447
1448
1449
1450
1451
1452
1453
1454
1455
1456
1457
1458
1459
1460
1461
1462
1463
1464
1465
1466
1467
1468
1469
1470
1471
1472
1473
1474
1475
1476
1477
1478
1479
1480
1481
1482
1483
1484
1485
1486
1487
1488
1489
1490
1491
1492
1493
1494
1495
1496
1497
1498
1499
1500
1501
1502
1503
1504
1505
1506
1507
1508
1509
1510
1511
1512
1513
1514
1515
1516
1517
1518
1519
1520
1521
1522
1523
1524
1525
1526
1527
1528
1529
1530
1531
1532
1533
1534
1535
1536
1537
1538
1539
1540
1541
1542
1543
1544
1545
1546
1547
1548
1549
1550
1551
1552
1553
1554
1555
1556
1557
1558
1559
1560
1561
1562
1563
1564
1565
1566
1567
1568
1569
1570
1571
1572
1573
1574
1575
1576
1577
1578
1579
1580
1581
1582
1583
1584
1585
1586
1587
1588
1589
1590
1591
1592
1593
1594
1595
1596
1597
1598
1599
1600
1601
1602
1603
1604
1605
1606
1607
1608
1609
1610
1611
1612
1613
1614
1615
1616
1617
1618
1619
1620
1621
1622
1623
1624
1625
1626
1627
1628
1629
1630
1631
1632
1633
1634
1635
1636
1637
1638
1639
1640
1641
1642
1643
1644
1645
1646
1647
1648
1649
1650
1651
1652
1653
1654
1655
1656
1657
1658
1659
1660
1661
1662
1663
1664
1665
1666
1667
1668
1669
1670
1671
1672
1673
1674
1675
1676
1677
1678
1679
1680
1681
1682
1683
1684
1685
1686
1687
1688
1689
1690
1691
1692
1693
1694
1695
1696
1697
1698
1699
1700
1701
1702
1703
1704
1705
1706
1707
1708
1709
1710
1711
1712
1713
1714
1715
1716
1717
1718
1719
1720
1721
1722
1723
1724
1725
1726
1727
1728
1729
1730
1731
1732
1733
1734
1735
1736
1737
1738
1739
1740
1741
1742
1743
1744
1745
1746
1747
1748
1749
1750
1751
1752
1753
1754
1755
1756
1757
1758
1759
1760
1761
1762
1763
1764
1765
1766
1767
1768
1769
1770
1771
1772
1773
1774
1775
1776
1777
1778
1779
1780
1781
1782
1783
1784
1785
1786
1787
1788
1789
1790
1791
1792
1793
1794
1795
1796
1797
1798
1799
1800
1801
1802
1803
1804
1805
1806
1807
1808
1809
1810
1811
1812
1813
1814
1815
1816
1817
1818
1819
1820
1821
1822
1823
1824
1825
1826
1827
1828
1829
1830
1831
1832
1833
1834
1835
1836
1837
1838
1839
1840
1841
1842
1843
1844
1845
1846
1847
1848
1849
1850
1851
1852
1853
1854
1855
1856
1857
1858
1859
1860
1861
1862
1863
1864
1865
1866
1867
1868
1869
1870
1871
1872
1873
1874
1875
1876
1877
1878
1879
1880
1881
1882
1883
1884
1885
1886
1887
1888
1889
1890
1891
1892
1893
1894
1895
1896
1897
1898
1899
1900
1901
1902
1903
1904
1905
1906
1907
1908
1909
1910
1911
1912
1913
1914
1915
1916
1917
1918
1919
1920
1921
1922
1923
1924
1925
1926
1927
1928
1929
1930
1931
1932
1933
1934
1935
1936
1937
1938
1939
1940
1941
1942
1943
1944
1945
1946
1947
1948
1949
1950
1951
1952
1953
1954
1955
1956
1957
1958
1959
1960
1961
1962
1963
1964
1965
1966
1967
1968
1969
1970
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2022
2023
2024
2025
2026
2027
2028
2029
2030
2031
2032
2033
2034
2035
2036
2037
2038
2039
2040
2041
2042
2043
2044
2045
2046
2047
2048
2049
2050
2051
2052
2053
2054
2055
2056
2057
2058
2059
2060
2061
2062
2063
2064
2065
2066
2067
2068
2069
2070
2071
2072
2073
2074
2075
2076
2077
2078
2079
2080
2081
2082
2083
2084
2085
2086
2087
2088
2089
2090
2091
2092
2093
2094
2095
2096
2097
2098
2099
2100
2101
2102
2103
2104
2105
2106
2107
2108
2109
2110
2111
2112
2113
2114
2115
2116
2117
2118
2119
2120
2121
2122
2123
2124
2125
2126
2127
2128
2129
2130
2131
2132
2133
2134
2135
2136
2137
2138
2139
2140
2141
2142
2143
2144
2145
2146
2147
2148
2149
2150
2151
2152
2153
2154
2155
2156
2157
2158
2159
2160
2161
2162
2163
2164
2165
2166
2167
2168
2169
2170
2171
2172
2173
2174
2175
2176
2177
2178
2179
2180
2181
2182
2183
2184
2185
2186
2187
2188
2189
2190
2191
2192
2193
2194
2195
2196
2197
2198
2199
2200
2201
2202
22

1 REFERENCES

- 2
- 3 Balligand, J.L., Kobzik, L., Han, X., Kaye, D.M.,
4 Belhassen, L., O'Hara, D.S., Kelly, R.A., Smith, T.W. and
5 Michel, T.
6 J. Biol. Chem., 1995 270 14582-14586
7
- 8 Beri, R.K. and Marley, A.E.
9 See, C.G., Sopwith, W.F., Aguan, K., Carling, D.,
10 Scott, J., and Carey, F.
11 Febs Lett, 1994 356 117-121
12
- 13 Bredt, D.S. and Snyder, S.H.
14 Proc. Natl. Acad. Sci. USA, 1990 87 682-685
15
- 16 Carling, D., Aguan, K., Woods, A., Verhoeven, A.J.M.,
17 Beri, R., Brennan, C.H., Sidebottom, C., Davidson, M.D. and
18 Scott, J.
19 J. Biol. Chem., 1994 269 11442-11448
20
- 21 Celenza, J.L. and Carlson, M.
22 Science, 1986 233 1175-1180
23
- 24 Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C.,
25 Busse, R., and Zeiher, A. M. (1999) Nature 399(6736), 601-5
26
- 27 Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J.,
28 Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A.,
29 and Sessa, W. C. (1999) Nature 399(6736), 597-601
30
- 31 Gallis, B., Corthals, G. L., Goodlett, D. R., Ueba, H.,
32 Kim, F., Presnell, S. R., Figeys, D., Harrison, D. G.,
33 Berk, B. C., Aebersold, R., and Corson, M. A. (1999) J Biol
34 Chem 274(42), 30101-8
35

- 1 Hardie, D.G. and Carling, D.
2 Eur J Biochem., 1997 246 259-273
3
4 Hayashi, T., Hirshman, M. F., Kurth, E. J., Winder, W. W.,
5 and Goodyear, L. J. (1998) Diabetes 47(8), 1369-73
6
7 Kemp, B.E. and Pearson, R.B.
8 Biochim. Biophys. Acta, 1991 1094 67-76
9
10 Kobe, B., Heierhorst, J., Feil, S.C., Parker, M.W.,
11 Benian, G.M., Weiss, K.R. and Kemp, B.E.
12 Embo. J., 1996 15 6810-6821
13
14 Kudo, N., Barr, A.J., Barr, R.L., Desai, S.,
15 Lopaschuk, G.D.
16 J. Biol. Chem., 1995 270 17513-17520
17
18 Matsubara, M., Titani, K. and Taniguchi, H.
19 Biochemistry, 1996 35 14651-14658
20
21 Michel, T. and Feron, O.
22 J. Clin. Invest., 1997 100 2146-2152
23
24 Michell, B.J., Stapleton, D., Mitchelhill, K.I.,
25 House, C.M., Katsis, F., Witters, L.A. and Kemp, B.A.
26 J. Biol. Chem., 1996 271 28445-28450
27
28 Michell, B. J., Griffiths, J. E., Mitchelhill, K. I.,
29 Rodriguez-Crespo, I., Tiganis, T., Bozinovski, S., de
30 Montellano, P. R., Kemp, B. E., and Pearson, R. B. (1999)
31 Curr Biol 12(9), 845-848
32
33 Mitchelhill, K.I., Michell, B.J., House, C.,
34 Stapleton, D., Dyck, J., Gamble, J., Ullrich, C.,
35 Witters, L.A., and Kemp, B.E.
36 J. Biol. Chem., 1997 272 24475-24479
37

- 1 Mitchelhill, K.I., Stapleton, D., Gao, G., House, C.,
2 Michell, B., Katsis, F., Witters, L.A. and Kemp, B.E.
3 J. Biol. Chem., 1994 269 2361-2364
4
5 **Mitchelhill, K.I. and Kemp, B.E. (1999) in: Protein**
6 **Phosphorylation: A Practical Approach, 2nd Ed., pp. 127-151**
7 **(Hardie, D.G., Ed.) Oxford University Press, Oxford.**
8 **"Phosphorylation site analysis by mass spectrometry".**
9
10 Moncada, S., and Higgs, A. (1993) N Engl J Med 329(27),
11 2002-12
12
13 Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda,
14 H., Kalka, C., Kearney, M., Chen, D., Symes, J. F.,
15 Fishman, M. C., Huang, P. L., and Isner, J. M. (1998) J
16 Clin Invest 101(11), 2567-78
17
18 Rodriguez-Crespo, I., Ortiz de Montellano, P.R.
19 Arch. Biochem. Biophys., 1996 336 151-156
20
21 Radomski, M. W., and Moncada, S. (1993) Adv Exp Med Biol
22 344, 251-64
23
24 Riddell, D.R. Graham A. Owen J.S. 1997 J. Biol. Chem 272,
25 89-95
26
27 Riddell D.R. and Owen J.S. (1999) Nitric Oxide and Platelet
28 Aggregation in Vitamins and Hormones 57, 25-48.
29
30 Ruan, J., Xie, Q., Hutchinson, N., Cho, H., Wolfe, G.C. and
31 Nathan, C.
32 J. Biol. Chem., 1996 271 22679-22686
33
34 Rudic, R. D., Shesely, E. G., Maeda, N., Smithies, O.,
35 Segal, S. S., and Sessa, W. C. (1998) J Clin Invest 101(4),
36 731-6
37

- 1 Salerno, J.C., Harris, D.E., Irizarry, K., Patel, B.,
2 Morales, A.J., Smith, S., Martasek, P., Roman, L.J.,
3 Masters, B., Jones, C.L., Weissman, B.A., Lane, P. *et al.*
4 J. Biol. Chem., 1997 272 29769-29777
5
6 Silvagno, F., Xia, H. and Bredt, D.S.
7 J. Biol. Chem., 1996 271 11204-11208
8
9 Stapleton, D., Guang, G., Michell, B.J., Widmer, J.,
10 Mitchelhill, K.I., Teh, T., House, C.M., Witters, L.A. and
11 Kemp, B.E.
12 J. Biol. Chem., 1994 269 29343-29346
13
14 Stapleton, D., Mitchelhill, K.I., Gao, G., Widmer, J.,
15 Michell, B.J., Teh, T., House, C.M., Fernandez, C.S., Cox,
16 T., Witters, L.A. and Kemp, B.E.
17 J. Biol. Chem., 1996 271 611-614
18
19 Stapleton, D.A., Woollatt, E., Mitchelhill, K.I.,
20 Nicholl, J.K., Fernandez, C.S., Michell, B.J.,
21 Witters, L.A., Power, D.A., Sutherland, G.R. and Kemp, B.E.
22 FEBS Lett., 1997 409 452-456
23
24 Stapleton, D., Woollatt, E., Mitchelhill, K.I.,
25 Nicholl, J.K., Fernandez, C.S., Michell, B.J.,
26 Witters, L.A., Power, D.A., Sutherland, G.R. and Kemp, B.E.
27 Febs Lett, 1997 411 452-456
28
29 Vavvas, D., Apazidis, A., Saha, A.K., Gamble, J.,
30 Patel, A., Kemp, B.E., Witters, L.A. and Ruderman, N.B.
31 J. Biol. Chem., 1997 272 13255-13261
32
33 Venema, R.C., Sayegh, H.S., Kent, J.D., Harrison, D.J.
34 J. Biol. Chem., 1996 271 6435-6440
35
36 Winder, W.W. and Hardie, D.G.
37 Am. J. Physiol., 1996 270 E299-E304

- 1
- 2 Zoche, M., Beyermann, M. and Koch, K.W.
- 3 Biol. Chem., 1997 378 851-857
- 4

CLAIMS:

1. A method of identifying modulators of AMPK-mediated activation of a nitric oxide synthase enzyme selected from the group consisting of eNOS, nNOS and nNOS μ , comprising
5 the step of testing putative modulators for their ability to increase or decrease phosphorylation of the enzyme, said increase or decrease depending on the calmodulin and calcium ion concentrations.

2. A method according to claim 1, in which the specific
10 phosphorylation of Ser-1177 is assessed in the presence of calcium and calmodulin.

3. A method of identifying modulators of AMPK-mediated inhibition of eNOS, comprising the step of testing a putative modulator for its ability to decrease or increase
15 AMPK-mediated phosphorylation of eNOS in the presence of limiting calcium ions.

4. A method according to claim 3, in which the specific phosphorylation of Thr-495 is assessed.

5. A method according to any one of Claims 1 to 4, in
20 which one or more of the following activities is additionally assessed:

- (a) Effect on smooth muscle contraction;
- (b) Effect on inotropic activity of the heart;
- (c) Effect on chronotropic activity of the heart; or
- 25 (d) Effect on platelet function.

6. A method according to any one of Claims 1 to 5, in which the modulator is an activator, as herein defined.

7. A method according to Claim 6, in which the activator promotes both glucose metabolism and fatty acid metabolism.

- 29 -

8. A method according to any one of Claims 1 to 5, in which the modulator is an inhibitor, as herein defined.

9. A method according to any one of Claims 3 to 8, in which the modulator acts preferentially on non-neuronal
5 cells.

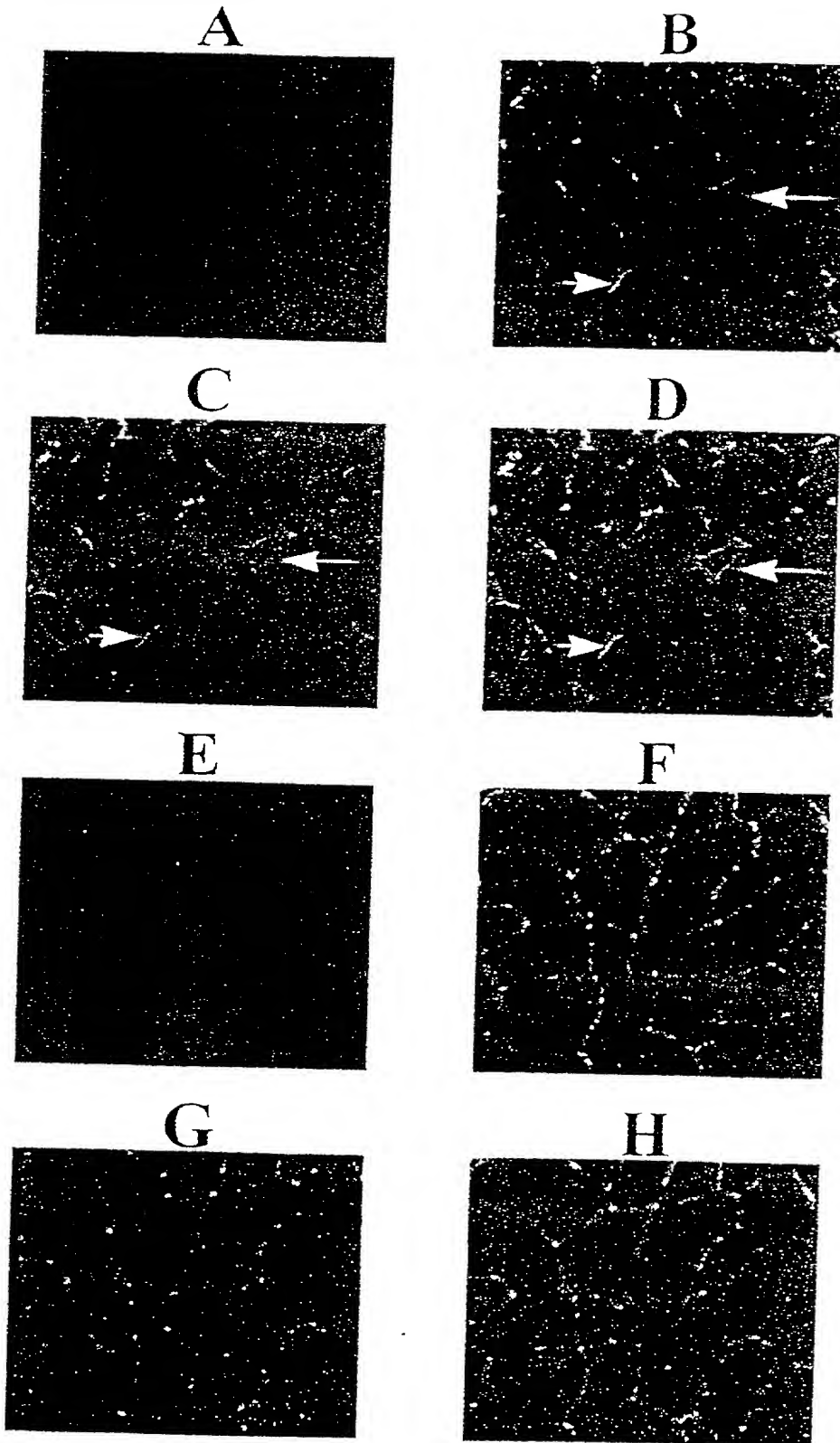
10. A method according to Claim 1 or Claim 2, in which the modulator promotes the dephosphorylation of Ser-1177 and inhibits eNOS activity.

11. A method according to Claim 3, in which the modulator
10 promotes the dephosphorylation of Thr-495 and stimulates eNOS activity.

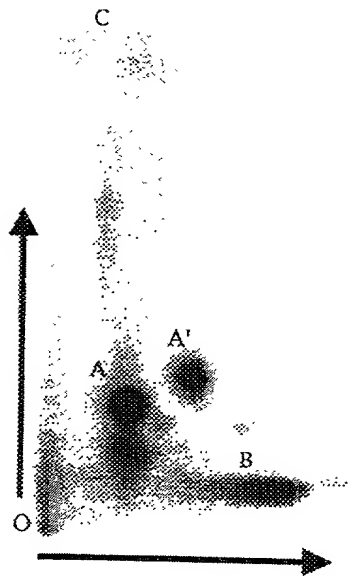
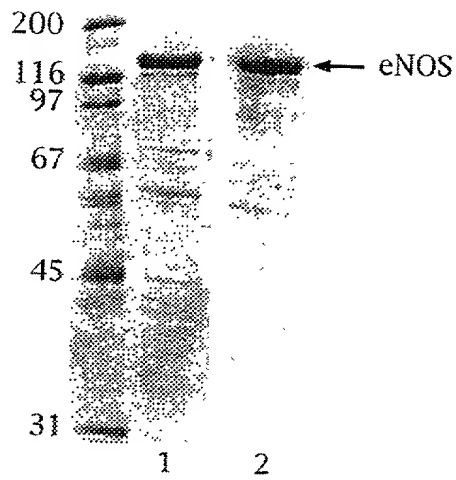
12. A method according to Claim 1 or Claim 2 in which the modulator promotes phosphorylation of nNOS or nNOS μ at Ser-1417.

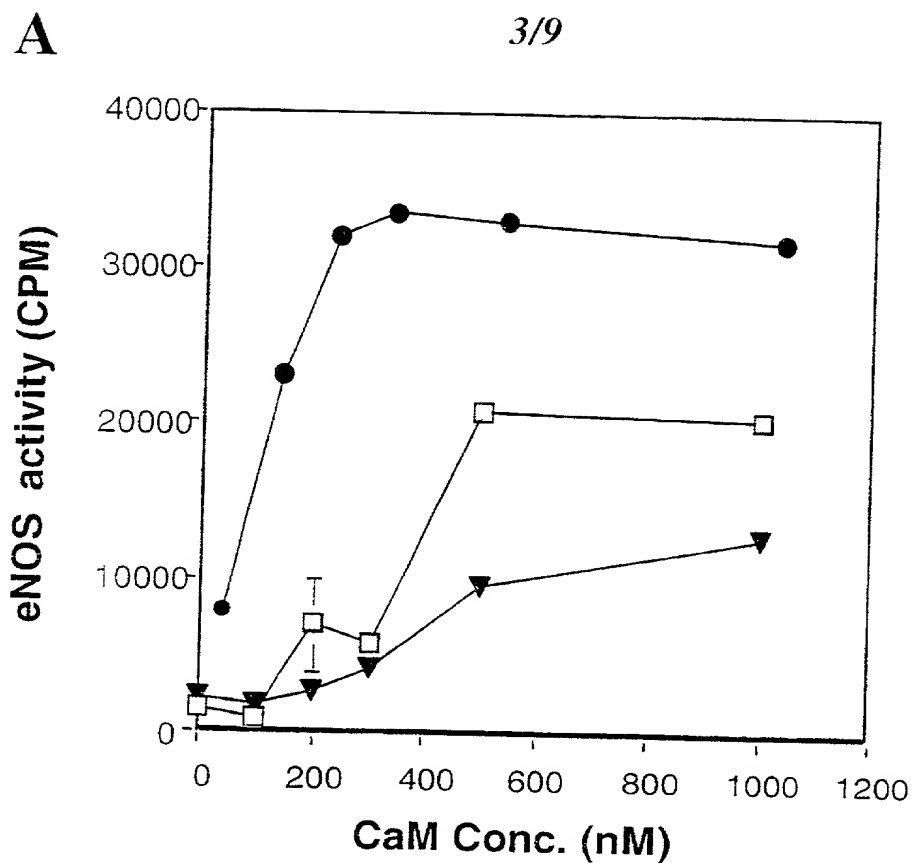
13. A method according to Claim 1 or Claim 2 in which the
15 modulator promotes dephosphorylation of nNOS or nNOS μ at Ser-1417.

1/9

*Figure 1*

2/9

*Figure 2*



eNOS + AMPK
+ Ca²⁺-CaM

eNOS + AMPK

A

●

○

A

B

●

○

Figure 3

4/9

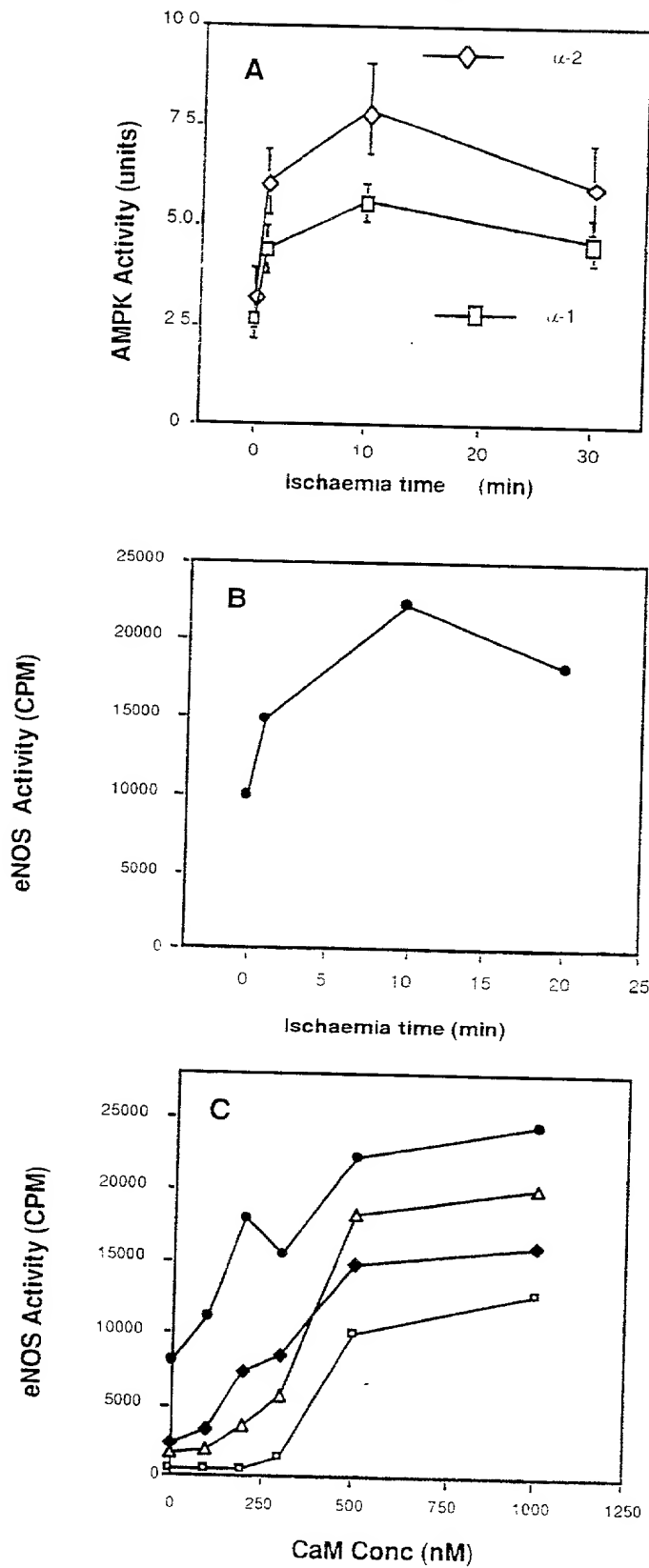


Figure 4

5/9

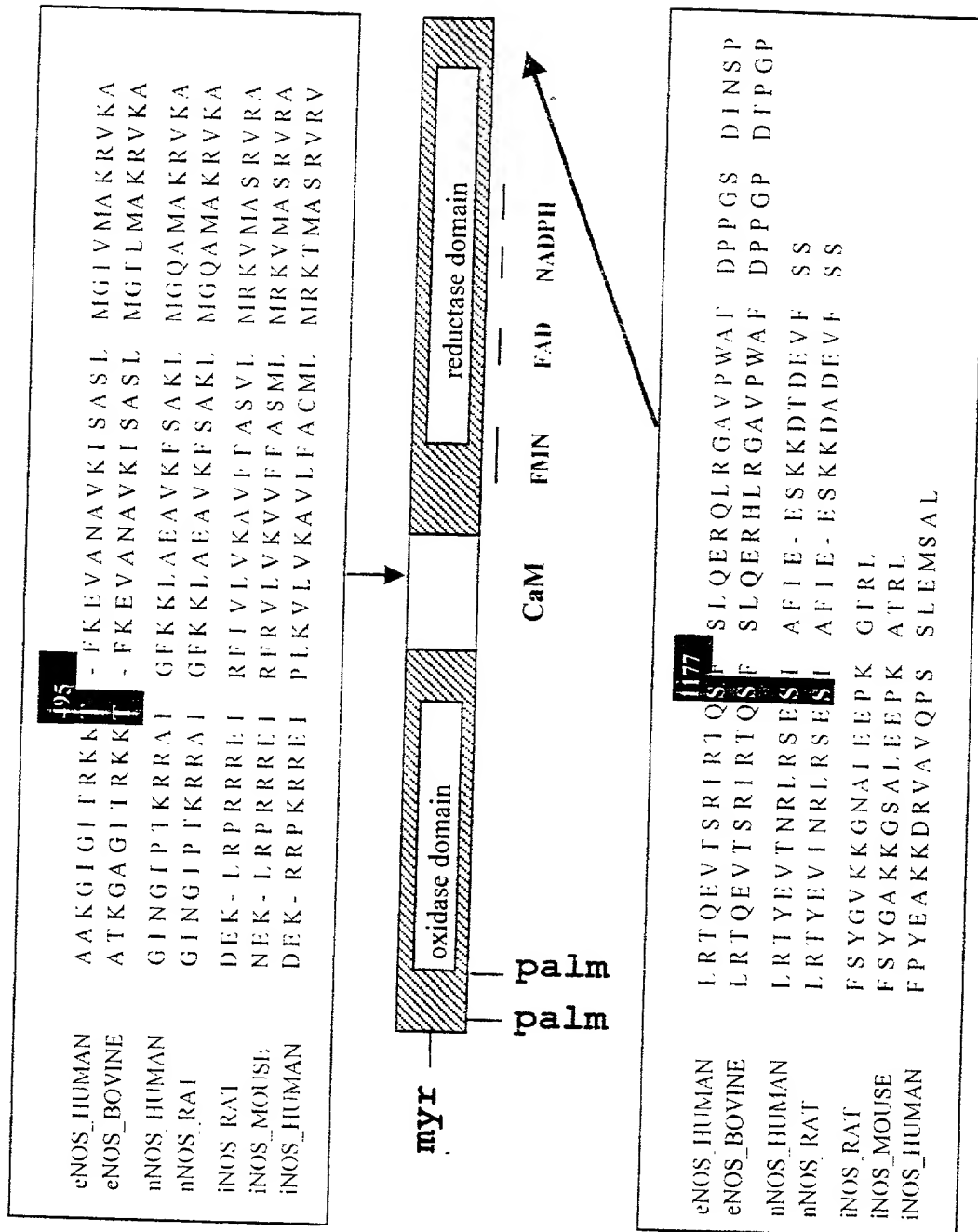


Figure 5

6/9

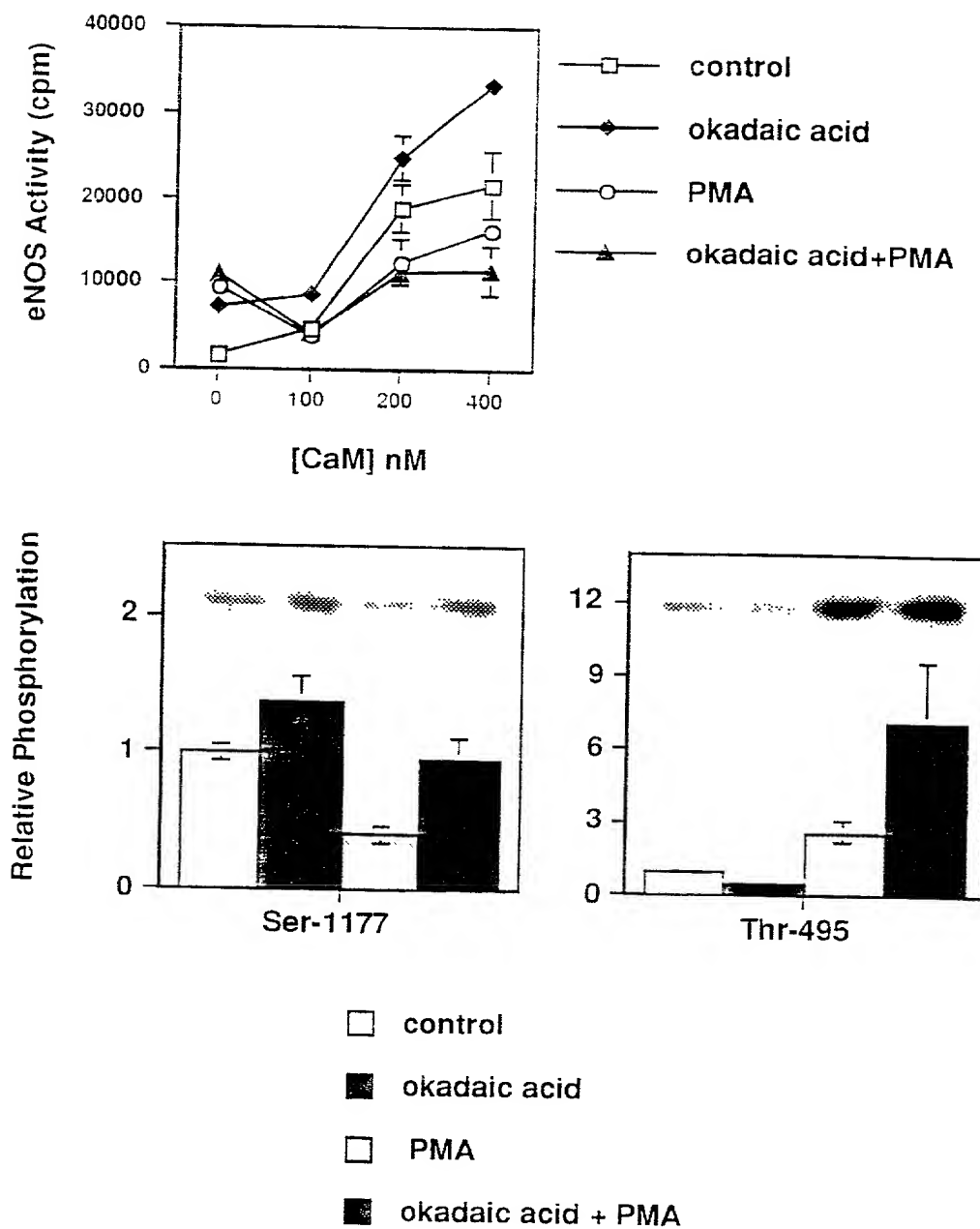
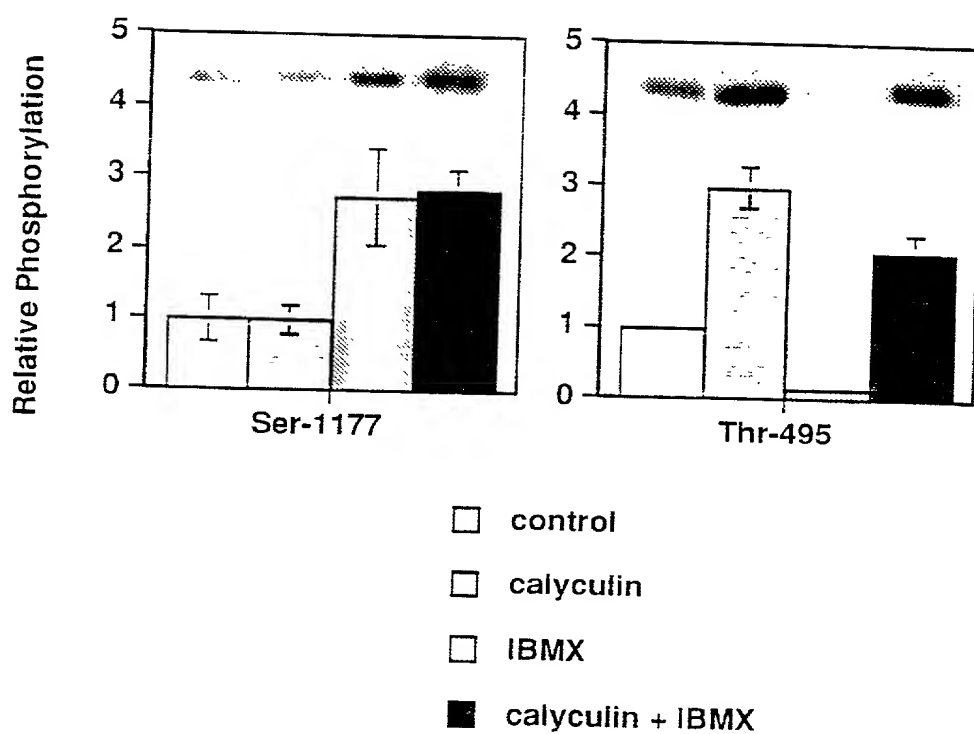
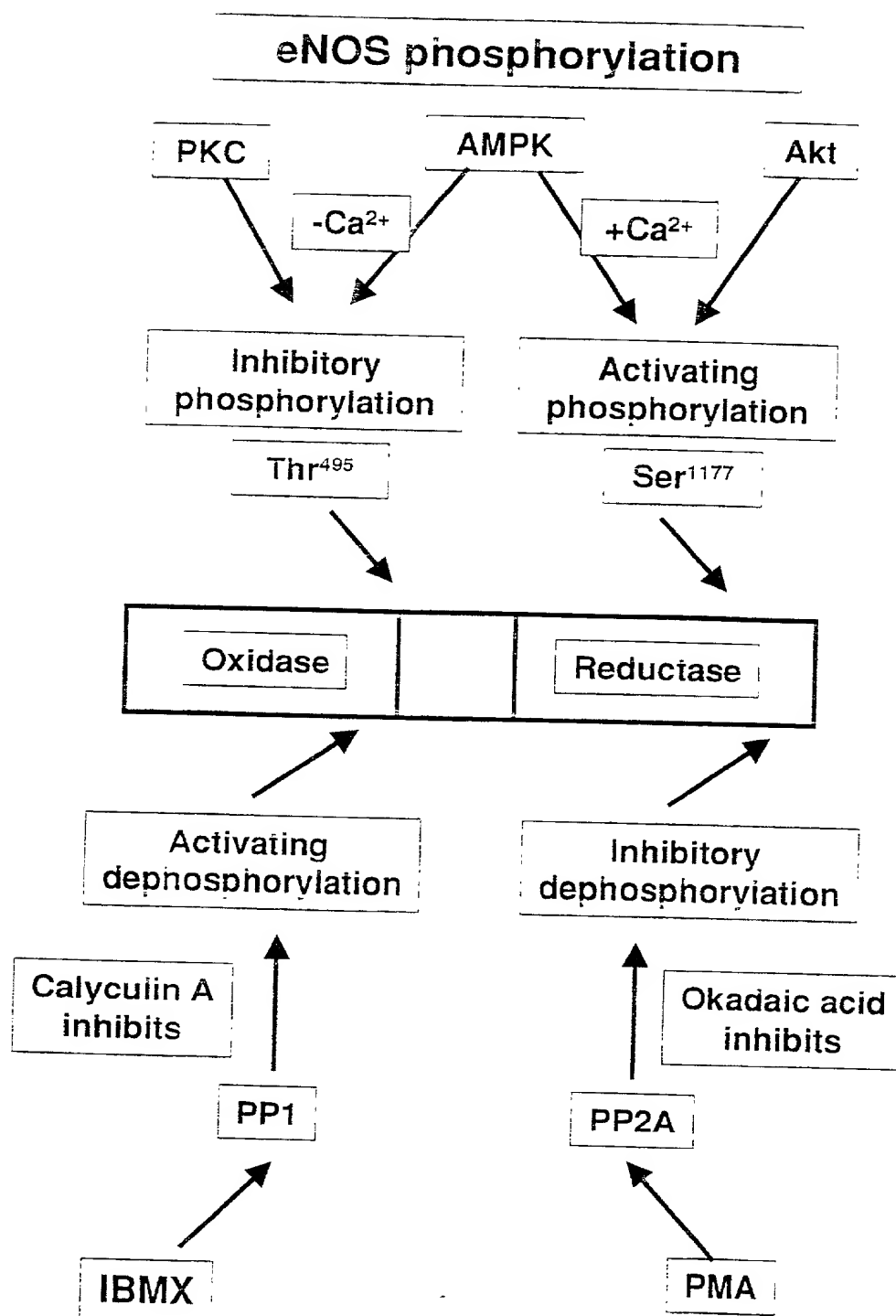


Figure 6

7/9

*Figure 7*

8/9

*Figure 8*

9/9

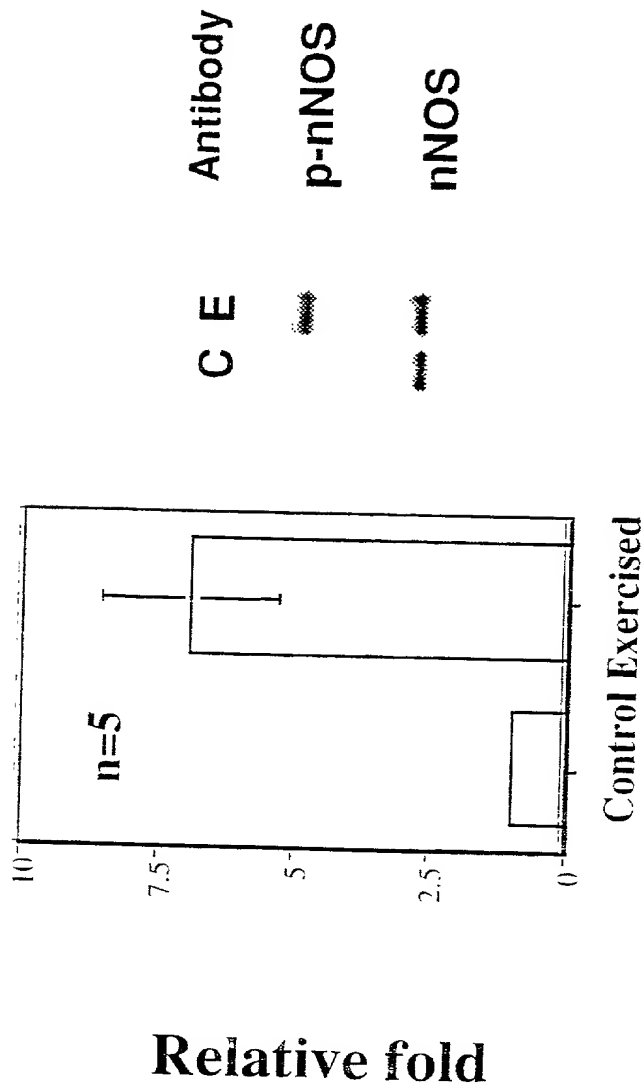


Figure 9

DECLARATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or the below named inventors are the original, first and joint inventors (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **Regulation of Nitric Oxide Synthase Activity**, the Specification of which:

☒ is attached hereto.
☐ was filed on _____ as Application Serial No. _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims.

I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability of the subject matter claimed in this application, as "materiality" is defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim priority benefits under Title 35, United States Code, § 119 and/or § 365 of any foreign application(s) for patent or inventor's certificate, PCT international application(s), and United States provisional application(s), listed below and have also identified below any foreign application for patent or inventor's certificate, PCT international application, or United States provisional application, having a filing date before that of the application on which priority is claimed:

PRIORITY APPLICATION(S)			Priority Claimed
PCT/AU99/00968	PCT	November 5, 1999	Yes
(Number)	(Country)	(Date Filed)	Yes/No
PP 6976	Australia	November 6, 1998	Yes
(Number)	(Country)	(Date Filed)	Yes/No

I hereby claim the benefit under Title 35, United States Code, § 120 and/or § 365 of any United States application(s) and PCT international application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose all information known to me to be material to patentability of the subject matter claimed in this application, as "materiality" is defined in Title 37, Code of Federal Regulations, § 1.56, which becomes available between the filing date of the prior application and the national or PCT international filing date of this application:

N/A		
(Application Serial No.)	(Filing Date)	(Status)
(Application Serial No.)	(Filing Date)	(Status)

I hereby direct that all correspondence and telephone calls be addressed to Shelley P.M. Fussey, Williams, Morgan & Amerson, P.C., 7676 Hillmont, Suite 250, Houston, Texas 77040, (713) 934-7000.

I hereby declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Inventor's Full Name:	David	Ian	Stapleton
Inventor's Signature:	David Stapleton		
Country of Citizenship:	Australia	Date:	14/11/00
Residence Address: (street, number, city, state, and/or country)	12 Mint Street, Wantirna, Victoria 3152 Australia AU X		
Post Office Address: (if different from above)			

Inventor's Full Name:	Zhiping	Chen
Inventor's Signature:	Zhiping Chen	
Country of Citizenship:	Australia AU X	Date: 14/11/00
Residence Address: (street, number, city, state, and/or country)	14 Eagle Rise, Lower Templestowe, Victoria 3107 Australia	
Post Office Address: (if different from above)		

Inventor's Full Name:	Belinda	Joyce	Michell
Inventor's Signature:	BJM Michell		
Country of Citizenship:	Australia	Date:	14/11/00
Residence Address: (street, number, city, state, and/or country)	12 Rose Avenue, Glen Waverley, Victoria 3150 Australia AU X		
Post Office Address: (if different from above)			

4-10

Inventor's Full Name:	Bruce	Ernest	Kemp
Inventor's Signature:	<i>B E Kemp</i>		
Country of Citizenship:	Australia	Date:	Nov 14 2000
Residence Address: (street, number, city, state, and/or country)	20 Kellett Grove, Kew, Victoria 3101 Australia <i>AUX</i>		
Post Office Address: (if different from above)			

5-10

Inventor's Full Name:	Kenneth	Ian	Mitchelhill
Inventor's Signature:	<i>K Mitchell</i>		
Country of Citizenship:	Australia	Date:	19th Nov 2000
Residence Address: (street, number, city, state, and/or country)	20 Oxford Street, Mount Waverley, Victoria 3149 Australia <i>AUX</i>		
Post Office Address: (if different from above)			